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Studies of Properties of the Liver Mitotic Stimulant (LMS) in Mouse Tumor Tissue [1]

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SUMMARY

A substance capable of increasing the liver mitotic activity was demonstrated in both the heated and unheated supernatant fraction of C3HBA mammary-tumor tissue. This liver mitotic stimulant (LMS) was heat-stable at 80° C for 20 minutes. The LMS could be stored dry in a vacuum or lyophilized. It was present in a variety of tumor types, was not strain-specific, and contained a high concentration of nucleic acids. Unheated normal tissue does not possess this LMS activity, but on heating at 80° C for 20 minutes the normal tissue becomes active. This strongly suggests the presence of a heat-labile LMS inhibitor in normal tissues and the absence of a similar inhibitor in cancer tissues.—J. Nat. Cancer Inst. 26: *525–531*, 1961.

MANY EFFECTS of tumors on the physiology of the tumor-bearing host have been noted (*1–6*).

One of these reported changes is an increase in the mitotic activity of the liver in mice (*7, 8*) and rats (*7*) with spontaneous and transplanted tumors. This increase in the liver mitotic rate has been demonstrated also in non-tumor-bearing mice treated with a presumably cell-free supernatant fraction of tumor tissue (*8*). In this instance the mitotic activity elicited was in direct relation to the amount of cancer extract administered, which suggested that a circulating factor (LMS) was released by the tumor.

In an attempt to characterize further the material in tumor tissue responsible for increased liver mitosis, the following study of the LMS was undertaken.

MATERIALS AND METHODS

Except when otherwise noted, the transplanted mouse mammary tumor C3HBA carried in strain C3H/He mice was used as the source of the liver mitotic stimulant.[3] Except in the studies of strain specificity, the

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[3] This tumor was kindly provided by Dr. Morris K. Barrett of the National Cancer Institute.

material prepared from this tissue was injected into 2- to 4-month-old female mice of strain C3H/He.

A representative portion of the left lobe of the liver was used in all studies. The liver tissue was fixed in 10 percent formalin, sectioned at 5 μ, and stained with hematoxylin and eosin.

In all studies the mitotic activity was established by counting the number of cells in mitosis in 25 microscopic fields at 500×.

Effect of colchicine.—Four-tenths ml of a preparation obtained by passing the C3HBA mouse mammary tumor through a tissue press was injected subcutaneously into 2- to 4-month-old strain C3H/He female mice. Forty-eight hours later the mice received intraperitoneal injections of 0.5 ml of various concentrations of colchicine, ranging from 0.8 to 0.00002 mg, in 0.85 percent saline. Six hours after the colchicine injections the animals were killed, and their livers were prepared and examined in the standard manner.

When the optimum concentration of colchicine was established, 0.5 ml of this concentration was administered to all test animals 6 hours before they were killed.

Heat stability.—The C3HBA mammary-tumor tissue or normal tissue to be tested was homogenized with 3 parts of 0.85 percent saline in a Waring blendor at 5° C for 2 minutes. The resulting homogenate was heated in a water bath at 80° C for 20 minutes, then centrifuged at 20,000 $\times$ g for 20 minutes. The supernatant was removed and 1.0 ml was injected intravenously into normal 2- to 4-month-old C3H/He female mice. Twelve, 24, and 48 hours after injection of the tumor supernatant, the mice were killed, and their livers were examined in the standard manner.

Effect of storage.—A C3HBA mammary-tumor-heated supernatant as prepared for the study of heat stability was divided into 5 portions. One aliquot was injected into mice immediately; a second was promptly placed in a deepfreeze at −20° C; a third was placed in an oven at 80° C; a fourth was dried *in vacuo* at room temperature; and a fifth was lyophilized. At the end of 3 days the frozen and the oven-heated aliquots were administered to mice. At the end of 7 days the vacuum-dried and the lyophilized material were reconstituted with distilled water to the original volume and injected into mice. All the preparations were injected intravenously in a dose of 1.0 ml into C3H/He female mice 2 to 4 months of age. Twenty-four hours later the animals were killed and their livers examined for mitosis.

Inhibition by normal tissues.—To determine whether or not unheated normal tissues possessed a factor capable of inhibiting the LMS of tumor tissue, C3H/He female mice 2 to 4 months of age received subcutaneous injections, in the back, of 0.4 ml of an undiluted brei of normal tissues prepared by passage of the tissue through a tissue press with 1 mm holes. The normal tissues included liver, spleen, and kidney from 2- to 4-month-old C3H/He female mice. Twenty-four hours later 1.0 ml of the supernatant fraction of a 25 percent C3HBA mammary-tumor brei that had been heated to 80° C for 20 minutes was injected intravenously. The

animals were killed 24 hours later, and their livers were examined for mitosis.

Tumor specificity.—For this portion of the study 0.8 ml of a 25 percent tumor brei, prepared by homogenization of the tumor tissue with 3 parts of 0.85 percent saline in a Waring blendor at 5° C for 2 minutes, was injected subcutaneously into C3H/He female mice 2 to 4 months old. The animals were killed 24 hours after the injection of the brei, and their livers were prepared and examined in the standard way. The tumors tested were all of C3H origin and included: 1) a methylcholanthrene-induced fibrosarcoma, HE 8971, in the 144th-transplant generation[4]; 2) two thorotrast-induced fibrosarcomas, H-36257, in the third-transplant generation and H-38290 in the sixth-transplant generation[5]; and 3) 3 spontaneous mammary tumors which arose in our breeding females, MT 31, MT 74, and MT 75, all in the second-transplant generation.

Strain specificity.—One ml of the heated supernatant prepared in the usual manner from C3HBA mammary-tumor tissue was injected intravenously into 2- to 4-month-old female mice of the following strains: BALB/cAnN, C3H/P, BRSUNT/N, AL/N, DBA/2JN, C57BL/6JN, DBA/P, and STR/N. The animals were killed 24 hours after the injection of the supernatant, and their livers were examined in the usual manner.

Spectrophotometry.—Samples of the heated supernatant fraction of C3HBA mammary tumor, prepared as described for the study on heat stability, were analyzed in a Cary spectrophotometer, model 114. Nitrogen and phosphorous determinations were also made on the same preparation. (We are indebted to Drs. Robert E. Greenfield and Vincent E. Price of the National Cancer Institute for the spectrophotometric analysis.)

RESULTS AND DISCUSSION

All the mice whose livers were studied received colchicine. Because colchicine arrests mitosis at the metaphase, it was anticipated that its administration would result in a more accurate measure of the increased number of mitoses occurring in the livers of the treated animals. Table 1 summarizes the results of administering various amounts of colchicine and shows that the optimum concentration for this study was the injection of 0.0002 mg per mouse 6 hours prior to killing in mice receiving 0.4 ml of a 100 percent C3HBA mammary-tumor brei.

The administration of colchicine alone in the concentrations tested did not affect the mitotic rate of liver tissue. There is no apparent explanation for the inhibition of the LMS by large amounts of colchicine.

Studies of the heat stability of the LMS, summarized in table 2, show that the LMS is heat-stable. At 12 and 24 hours after the intravenous administration of the supernatant fraction of a tumor brei heated to 80° C for 20 minutes, an increase in the liver mitotic activity was apparent. At 48 hours this effect was markedly decreased. A comparable increase

[4] This tumor was kindly provided by Dr. Walter E. Heston of the National Cancer Institute.
[5] These tumors were kindly provided by Dr. Richard L. Swarm of the National Cancer Institute.

TABLE 1.—Effect of various concentrations of colchicine on the liver mitosis of C3H/He mice treated with 0.4 ml of 100 percent C3HBA mammary-tumor brei

Colchicine (mg)	Tumor brei (ml)	Number of determinations	Average liver mitosis
None	0.4	4	12.2
0.8	0.4	4	0
0.4	0.4	4	0
0.2	0.4	13	0.6
0.02	0.4	7	6.8
0.002	0.4	13	15.7
0.0002	0.4	11	67.6
0.00002	0.4	6	22.0
0.8	None	4	0
0.4	None	4	0
0.2	None	6	0
0.02	None	2	0
0.002	None	2	0
0.0002	None	3	0

TABLE 2.—Effect of heat at 80° C for 20 minutes on the liver mitotic stimulant in the supernatant fraction of C3HBA mammary tumor and normal tissues as tested by intravenous injection of 1 ml into C3H mice

Tissue	Treatment of tissue	Hours after injection liver removed	Number of determinations	Average liver mitosis
C3HBA mammary tumor	No heat	24	3	10
"	Heat	12	3	12
"	"	24	27	15
"	"	48	9	4
C3H liver	No heat	24	15	0.8
"	Heat	24	14	13
C3H blood	"	24	5	11
" spleen	"	24	6	6
" kidney	"	24	6	10

in the liver mitotic activity is produced 24 hours after the injection of an unheated tumor supernatant fraction.

In contrast, the supernatant fraction prepared from normal liver tissue produced no increase in the liver mitotic activity. However, when this normal liver supernatant was heated to 80° C for 20 minutes, it also was capable of stimulating liver mitotic activity similar to that observed for tumor extract. As shown in table 2, the supernatants of normal blood, spleen, and kidney, when heated, also elicited an increase in the liver mitotic rate.

In view of this, speculation about the apparent effect of heat on normal tissue is inevitable. The explanation that comes most readily to mind suggests that normal tissues contain a substance capable of stimulating liver mitosis and that this substance, like the cancer-associated LMS, is heat-stable. And, that in addition to this, the normal tissues also contain a heat-labile inhibitor that is absent from cancerous tissues. If this explanation is valid, its possible relation to the process of carcinogenesis is interesting.

Along the same line, it is also of interest that the LMS of a cancer supernatant fraction is inhibited if the intravenous injection of the heated

TABLE 3.—Effect of the subcutaneous injection of 0.4 ml of a 100 percent normal-tissue brei upon the LMS effect of 1.0 ml of C3HBA mammary-tumor-heated supernatant injected intravenously 24 hours later

Material injected	Number of determinations	Mitosis
C3HBA intravenously	6	24
C3HBA intravenously + liver brei subcutaneously	6	0
Liver brei subcutaneously	6	0
C3HBA intravenously + spleen brei subcutaneously	6	0
Spleen brei subcutaneously	6	0
C3HBA intravenously + kidney brei subcutaneously	6	0
Kidney brei subcutaneously	6	0

supernatant fraction of a C3HBA mammary tumor is preceded by the subcutaneous injection of unheated normal tissue brei (table 3). Apparently the heat-labile inhibitor of normal tissue is also capable of inhibiting cancer-tissue activity when the two are administered to the same animal. The present study was not designed to show whether this inhibitor acted directly upon the LMS or whether its effect was mediated by some host response. Nor is it entirely clear why the hosts' own normal tissues, presumably capable of elaborating a similar inhibitor, do not inactivate the injected LMS.

Since it would be a distinct advantage from several points of view to be able to prepare and store large amounts of test material, several methods of preservation were tried. Table 4 summarizes the results. The LMS activity of the preparation was lost when stored either in the frozen state or in an oven at 80° C. The LMS activity was retained if the material was stored in a dehydrated state in a vacuum, or lyophilized. This is suggestive of destruction by a catabolic enzyme, though such an enzyme would have to be remarkably heat-stable, as the preparations were heated to 80° C before storage.

Although no completely comprehensive survey of all possible tumors was undertaken, the evidence obtained with several sarcomas and several mammary tumors (table 5) strongly suggests that the presence of an LMS is not unique to one histologic type tumor or to tumors of only one species (7).

TABLE 4.—Effect of storage in frozen state, at high temperatures, lyophilized, and dried *in vacuo* at room temperature on the LMS activity of C3HBA mammary-tumor extract

Material tested	Number of examinations	Average liver mitosis
Freshly prepared	3	30
Deepfreeze, −20° C, for 3 days	6	4
Oven 80° C for 3 days	3	2
Lyophilized and stored for 7 days	9	32
Dried *in vacuo* and stored for 7 days	3	25

TABLE 5.—Liver mitotic stimulating effect of 0.8 ml of a 25 percent brei preparation from several C3H tumors injected subcutaneously into C3H mice—livers were removed 48 hours later

Tumor	Number of examinations	Average liver mitosis
HE 8971	9	21
H 36257	3	9
H 38290	3	7
MT 31	3	31
MT 74	3	37
MT 75	3	19

Eight different strains or substrains of mice were given intravenous injections of the supernatant fraction of C3HBA mammary tumor heated to 80° C for 20 minutes to determine whether differences among the strains were detectable and what effect the LMS obtained from one strain would have when injected into another. Table 6 shows that the LMS factor is not strain-specific, for all strains tested showed some mitotic increase.

The spectrophotometric analysis of the heated supernatant from C3HBA mammary tumor revealed a single peak at 2600 A. This suggests that the heated supernatant was a nucleic acid material. The possibility that the nucleic acid concentration was sufficiently high to mask the presence of small amounts of protein cannot be excluded, however. That this is a very likely possibility is suggested by the results of the nitrogen and phosphorous determination. The N/P ratio was 3.64, about 2 times what it would be for a pure nucleic acid preparation. We conclude therefore that the LMS effect of the heated supernatant is associated with a high nucleic acid concentration, but the active material in the supernatant is not necessarily nucleic acid.

TABLE 6.—Effect of intravenous injection of 1.0 ml of a 25 percent C3HBA mammary-tumor-homogenate-heated supernatant on the liver mitosis of various strains of mice 24 hours after injection

Strain of mouse	BALB/cAnN	C3H/P	BRSUNT/N	AL/N	DBA/2JN	C57BL/6JN	DBA/P	STR/N
Number of determination	21	27	10	11	10	9	10	9
Liver mitotic activity	11	5	11	4	7	3	9	7

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Antigenicity of Pooled Human Malignant and Normal Tissues by Cytoimmunological Technique. III. Distribution of Tumor Antigen [1, 2]

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SUMMARY

Horse antiserum to pooled human carcinoma preparations displayed toxicity for monolayer cultures of HeLa cells. This toxicity was no longer present after absorption of the serum by human carcinomas. Each of 60 individually tested carcinomas absorbed approximately the same amount of toxic material. The relative amount of absorbing antigen in these tumors showed a standard deviation of ± 30 percent. Preparations from established cell strains, HeLa (Gey), Detroit-6 (Berman and Stulberg), conjunctiva (Chang), and heart (Girardi), also were able to remove the toxicity from the antiserum. Thus, 60 human carcinomas and atypical cells of established tissue-culture lines possessed a common antigen which was pertinent for the initiation of cytotoxic manifestations with the exposure *in vitro* of atypical cells to the horse antiserum. This antigen presumably constitutes a part of the plasma membrane.— J. Nat. Cancer Inst. 26: 533–545, 1961.

A SUBSTANTIAL amount of data is accumulating which indicates that human carcinoma tissue exhibits antigenicity different from that of normal tissues.

Evidence for characteristic tumor antigenicity was found by cell culture *in vitro* and immunochemical methods by Björklund and Björklund (*1*). The antigenic properties were connected with insoluble and heat-labile lipoprotein, presumably a constituent part of the plasma membrane (*2*). Toolan reported that immune serums to different tumors but not to normal tissues could inhibit tumor cell growth, and that the pertinent antigenicity was carried by an insoluble part of the tumor cells, possibly the cell membrane (*3*). Southam, Moore, and Rhoads in experiments on human volunteers found that injections of viable human cancer cells in healthy humans resulted in accelerated destruction of subsequently injected cancer cells of the *same or other* type (*4, 5*). In preliminary experiments, pretreatment with normal amnion cells or fibroblasts did not accelerate the destruction of subsequently injected

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cancer cells (*6*). These findings indicate that tumor cells share some antigenic properties which are absent from normal cells.

Based upon extensive studies, Zilber (*7*) concluded that the antigenic structure of cells of human tumors is distinct from that of normal cells. The antigen could be adsorbed on red cells. Gorodilova and Shershul'skaia (*8*) studying anaphylactic reactions in guinea pigs reached the same conclusion. Animals immunized with extracts of human carcinoma tissue, after desensitization with corresponding extracts of normal tissues, still reacted with the *same or other* carcinoma preparations. In 1929 Witebsky reported that a lipide tumor antigen could be demonstrated by complement fixation (*9*, *10*). A lipide haptene resembling Witebsky's alcoholic extract was shown by Björklund, Lundblad, and Björklund (*2*) by an entirely different method: demonstration of capacity to absorb cytotoxic antibodies.

Working with animal tumors, Pressman and Day reported that antibodies, prepared against Murphy-Sturm lymphosarcoma and labeled with radioactive iodine, localize in the Walker carcinosarcoma 256, the Novikoff hepatoma, and the Murphy-Sturm lymphosarcoma. Antibodies prepared against normal tissues either did not localize in tumors, or did so only slightly (*11*). Mohos and Kidd described an antibody that reacted specifically with a distinctive constituent of Brown-Pearce carcinoma cells and was able to suppress growth of this carcinoma *in vivo* (*12*). Preston and Henegar showed that rats with regressed Bagg's lymphosarcoma possessed a specific antibody against the tumor (*13*). Bale, Spar, and Goodland observed a preferential localization of radioactive iodine-labeled antitumor antibodies in the Murphy-Sturm lymphosarcoma as compared to any other tissue, including blood (*14*). A proportion of these antibodies, however, seemed to be directed against fibrin (*15*). Ambrose *et al.* (*16*, *17*) reported that malignant cells possess a high negative electric charge as compared to normal cells. These findings contribute important information and, interpreted as a whole, permit us to recognize properties that are typical of tumor cells.

In preliminary studies, the characteristic tumor antigenicity, described by Björklund and Björklund (*1*) was found in a number of different human carcinomas (*18*). The distribution of this antigenicity was determined in absorption experiments and the results were assayed in cell cultures *in vitro*. The applicability of the preliminary results to a larger number of carcinomas was then sought. The present report records observations on 60 individual human tumors and 4 established cell strains of human origin.

MATERIALS AND METHODS

Antigen.—Carcinomas from humans were obtained from the autopsy room. In accordance with procedures described elsewhere (*1*, *2*), the carcinomas were prepared by homogenization, ether extraction, lyophili-

zation, and ball-mill grinding at −70° C. The insoluble, heat-labile residue from each carcinoma was used separately.

The following types of carcinoma were used. The number of individual specimens of each type is in parentheses:

Cancer of the ovary (2)
Cancer of the stomach (5)
Cancer of the lung (13)
Cancer of the bladder (3)
Cancer of the uterus (3)
Cancer of the prostate (2)
Cancer of the colon (2)
Cancer of the breast (2)
Cancer of the liver (1)
Cancer of the biliary duct (1)
Hypernephroma (2)
Liver metastasis of cancer of the ovary (2)
Liver metastasis of cancer of the breast (4)
Liver metastasis of cancer of the rectum (2)
Liver metastasis of cancer of the lung (6)
Liver metastasis of cancer of the bladder (1)
Liver metastasis of cancer of the stomach (2)
Liver metastasis of cancer of the coecum (1)
Liver metastasis of cancer of the colon (2)
Liver metastasis of cancer of the pancreas (1)
Liver metastasis of granulosa-cell tumor of the ovary (1)
Cancer of the rectum with metastasis (1)
Cerebral metastasis of cancer of the stomach (1)

A total of 60 preparations was made from 60 individuals with 16 types of carcinoma. Thirty-seven preparations from the same number of individuals were made from primary tumors of 12 types of carcinoma. Twenty-three preparations from 23 individuals were made from liver metastases of 11 types of carcinoma. Seven types of tumors were represented by primary and metastatic growth. Each diagnosis was confirmed at autopsy and verified by microscopic examination of stained preparations.

Antiserum.—Serum was obtained from a horse immunized over a period of 6 years by subcutaneous injections of crude antigen from pooled human carcinomas, as previously described (*1*).

Cell cultures and cytotoxicity reaction.—Monolayer cell cultures of HeLa (Gey), Detroit-6 (Berman and Stulberg), conjunctiva (Chang), and heart (Girardi) were grown in stationary pyrex tubes, 150 × 15 mm, inclined at an angle of 5°. The stock cultures were grown in stationary flasks in 20 to 40 percent pooled, inactivated, human serum and 60 to 80 percent of balanced salt solution (BSS) (Simms X-7) or Parker 199. Penicillin 200 u/ml and tetracycline 25 γ/ml were used throughout.

Each tube culture was initiated on day 1 by the introduction of 0.5 ml of medium containing 100,000 cells/ml. On day 3 the medium was removed as completely as possible with Pasteur pipettes connected to water suction. Triplicate series of tubes were set up for each dilution of immune serum in medium. To each culture was added 0.5 ml of a dilution

of 1:10, 1:20, 1:40, and 1:80. After the cell cultures had been inoculated they were incubated at 35° C for 20 to 22 hours. At the end of this period each culture was examined microscopically (magnification: 6 × 12).

When monolayer cultures of HeLa cells had been exposed to serum containing specific antibodies, characteristic and reproducible changes occurred. The cell layers underwent retraction, clumping, and eventually lysis, which actually consisted of fragmentation of the cells.

The degree of cytotoxicity was recorded as −, +, ++, +++, or complete lysis (figs. 1 to 4). The details and the kinetics of this reaction had been previously studied (*1*). The sequence of events during immune lysis of HeLa cells was recorded by time-lapse cinemicrography through a phase-contrast microscope.

After examination of the cultures microscopically, the medium was replaced by fresh medium and regrowth, if any, was permitted and recorded after a period of 20 to 22 hours.

Absorption of antiserum by antigen.—This technique has been described in detail in previous reports (*1, 2*). In principle, the procedure was as follows: Horse antiserum to pooled human carcinoma preparations displayed cytotoxicity for monolayer cultures of HeLa cells. After absorption of the serum by antigen from carcinoma preparations, toxicity was no longer discernible. The relative amount of this antigen present in individual tumors was calculated from the amount of lyophilized antigen required for complete absorption of a standard immune serum. The reciprocal of this amount represented the relative antigenicity.

Portions of 2 ml of immune serum were mixed with 2.5, 15, 25, 37.5, 50, 62.5, and 75 mg of antigen per ml of serum, respectively, and permitted to stand overnight at 0° C. After centrifugation at 17,000 × *g* for 30 minutes at 0° C, the supernatants were passed through G 5 M filters and used in cell cultures. Preimmunization serum was used for control purposes and treated in the same way as the immune serum.

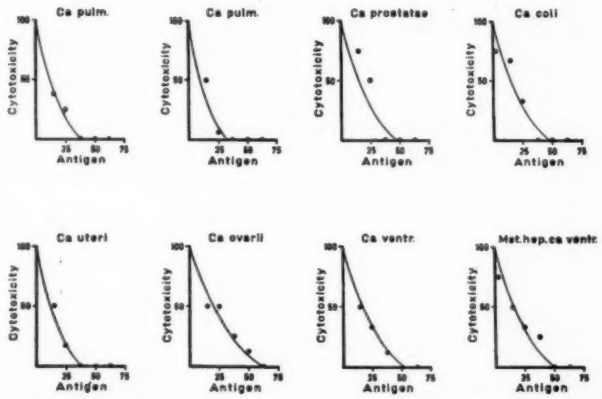
Absorption curve.—Cytotoxicity was kept a function of antibody concentration. Therefore, a decrease of antibody concentration as a consequence of specific absorption was recognized by a lowered serum cytotoxicity. By comparing the effect of dilution series of absorbed immune serum with dilution series of untreated immune serum, we could make quantitative interpretations of the effect of absorption. In this way absorption curves could be obtained, where the serum cytotoxicity was plotted against the quantity of antigen used for absorption. As the serum cytotoxicity was a function of antibody concentration, the curve visualized the removal of specific antibody by antigen or immunological absorption.

EXPERIMENTS AND RESULTS

Absorption of standard horse anti-human cancer serum by antigens (*see* "Materials and Methods") from 60 individual human carcinomas in each instance rendered the serum nontoxic to monolayer cultures of

HeLa cells. From previous work (*2*) it was known that absorption of the same serum by 15 different, normal tissue preparations did not remove the cytotoxicity. Controls with normal horse serum absorbed with the tumor preparations were invariably nontoxic. Therefore it could be concluded that cytotoxicity-absorbing antigen was present in each of the 60 individual tumors.

The absorption curves elucidated the interaction between serum and each tumor, and the amounts required for complete absorption were determined in each case. The mean of the reciprocal of these amounts, representing the relative antigenicity, showed a standard deviation of ± 30 percent. Some typical examples of individual absorption curves are shown in text-figure 1.



TEXT-FIGURE 1.—Absorption of horse antihuman cancer serum by individual carcinomas. *Cytoxicity* in percentage of untreated serum. *Antigen* in mg/ml of immune serum.

These experiments demonstrated that an antiserum prepared toward several hundred carcinomas of human origin could be made nontoxic to HeLa cells by absorption with antigen from individual human carcinomas.

Monolayer cell cultures of strains Detroit-6, conjunctiva, and heart were then substituted for HeLa. Horse anti-human cancer serum was absorbed with a preparation from one carcinoma (a lung cancer) and serial dilutions were added to cultures of the three strains. No toxicity was observed which indicated that after absorption with an individual carcinoma preparation the "polyvalent" antiserum had also lost toxicity against three established lines of atypical cells (table 1).

Absorption of the immune serum with HeLa cells was next undertaken. The cells from monolayer cultures in stationary bottles containing 50 ml of medium (60% Parker 199 + 40% human serum) were removed from the glass by a 1:5000 solution of Versene (Merck & Co., Inc., preparation, Titriplex) in Dulbecco's phosphate-buffered saline, part

TABLE 1.—Effect of absorption of horse antihuman cancer serum (immune serum)

Serum	Absorbing antigen	Cells used for cytotoxic reaction	Cytotoxicity
Immune		HeLa	+*
"		Detroit-6	+
"		Conjunctiva	+
"		Heart	+
"	Pooled human carcinomas	HeLa	+†
"	Individual carcinomas (60)	"	−
"	Detroit-6, conjunctiva, or heart cells	"	−
"	Human lung cancer	Detroit-6	−
"	" " "	Conjunctiva	−
"	" " "	Heart	−
"	HeLa cells	Detroit-6	−
"	" "	Conjunctiva	−
"	" "	Heart	−
Preimmunization	Human carcinomas	HeLa	−
"	" "	Detroit-6	−
"	" "	Conjunctiva	−
"	" "	Heart	−
"	HeLa, Detroit-6, conjunctiva, or heart cells	HeLa	−
"	" " " "	Detroit-6	−
"	" " " "	Conjunctiva	−
"	" " " "	Heart	−

* + Indicates complete cytolysis.
† − Indicates no visible reaction.

A. The cells were counted in a Buerker Chamber and washed 3 times with BSS (Simms X-7). Antiserum absorbed with these cells, in a preparation of 125 million cells/ml of serum, was no longer toxic to monolayer cultures of Detroit-6, conjunctiva, or heart cells. The toxic reaction was completely abolished, which showed that the "polyvalent" antiserum against several hundred human carcinomas could be exhausted by antigens from one established strain of atypical cells; it then was no longer reactive with three other established strains of atypical cells (table 1). The data show that these strains possess in common antigenic receptors pertinent to the cytotoxic reaction. A study of a larger number of cell strains is in progress.

The same experiment, but reversed, was also performed. Antiserum was absorbed with Detroit-6, conjunctiva, and heart cells separately and tested against HeLa cells. The results were the same as before; toxic effects were no longer discernible (table 1).

In earlier work, fractionation of the human carcinoma material had revealed that the cytotoxicity-absorbing antigen resided in the insoluble residue (*2*). This was true also for the material obtained from cell cultures *in vitro*. By extraction, the dry weight of the cells could be reduced by 50 percent with unimpaired antigenicity. High specific activity was observed in the slowly sedimenting material. This finding is in agreement with earlier results (*2*) and also with the experience of Toolan (*3*).

DISCUSSION

Unless certain precautions are taken, the cytotoxicity-absorbing antigen as described here cannot be demonstrated. The antigen can neither be exposed to prolonged heating above 40° C, nor treated with proteolytic enzymes, nor ground for hours at temperatures much above −70° C. All attempts to bring the complete antigen into true solution have failed. Furthermore, the antigen possesses weak immunizing properties; immunization periods are required which are longer than those usually used. These and other pertinent physicochemical data on the nature of the cytotoxicity-absorbing antigen have already been reported (*1*, *2*). It should be stressed, however, that without such data it would be difficult to perform meaningful experiments with the actual antigen.

Preliminary observations indicated that the antigen is widely distributed in human carcinomas (*18*). The present study adds weight to these findings by revealing that each of 60 individual carcinomas contains a similar or identical antigen. Quantitatively measured in relative units the antigen varied within the carcinoma population by a standard deviation of only ± 30 percent.

Interesting was the finding that antiserum to several hundred carcinomas could be made nontoxic to HeLa cells by absorption with individual carcinomas or with cells of established strains. These results indicate that the atypical cells contain an antigen which is essential for initiation

of the cytotoxic events. Antibodies to this antigen seem to "trigger" cytotoxic manifestations when made to react with atypical cells in an environment which contains accessory factors required for this type of cytolysis.

In previous work a large number of *soluble* antigens from a variety of human tissues, including carcinomas, were studied by gel-diffusion technique (*19*, *20*). None of these antigens, however, were able to absorb the cytotoxicity from horse anti-human cancer serum. Still other antigens were demonstrated in mitochondria by means of quantitative precipitation of antibody nitrogen from immune serum by fractions obtained at differential centrifugation of malignant cells (*21*). These antigens which were insoluble also were devoid of the capacity to absorb cytotoxicity.

Thus, the experimental evidence permits us to visualize a complex antigenic pattern of atypical cells. This pattern can be understood if one assumes that the antigenicity of pertinence for the cytotoxic reaction is an integrated part of the plasma membrane.

The findings reported in this paper, concerning the distribution of the cytotoxicity-absorbing antigen, attest to the validity of the original concept (*1*) that, when large numbers of carcinomas are pooled, individual tissue antigens are diluted out, whereas a postulated antigen common to these tumors would be reinforced, from a relative point of view.

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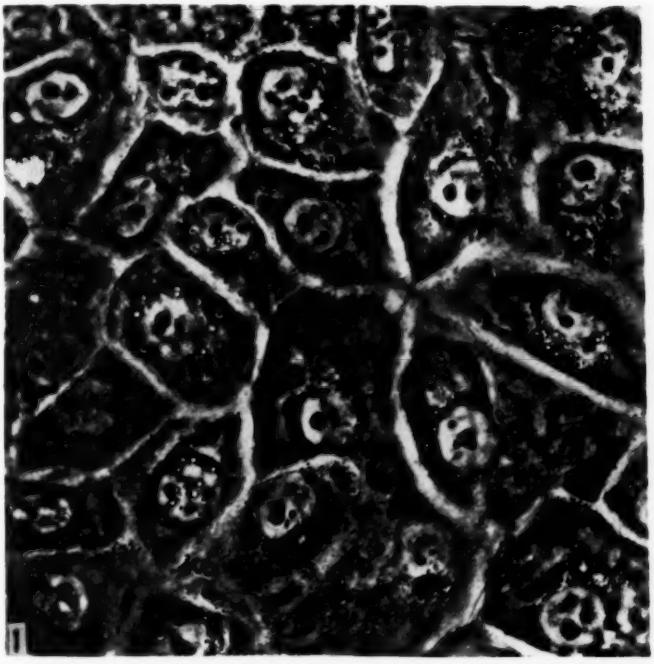
Figures 1 through 4 are phase-contrast micrographs of HeLa cells exposed to medium without and with horse antihuman cancer antibodies. 5 × 4

PLATE 76

FIGURE 1.—HeLa cell monolayer in medium (40% human serum, 60% Simms BSS, streptomycin 100 γ/ml, tetracycline 25 γ/ml).

FIGURE 2.—Retraction and some clumping of cells 20 hours after exposure to horse antibodies diluted 1 : 80 in medium. Reaction is recorded as ++.



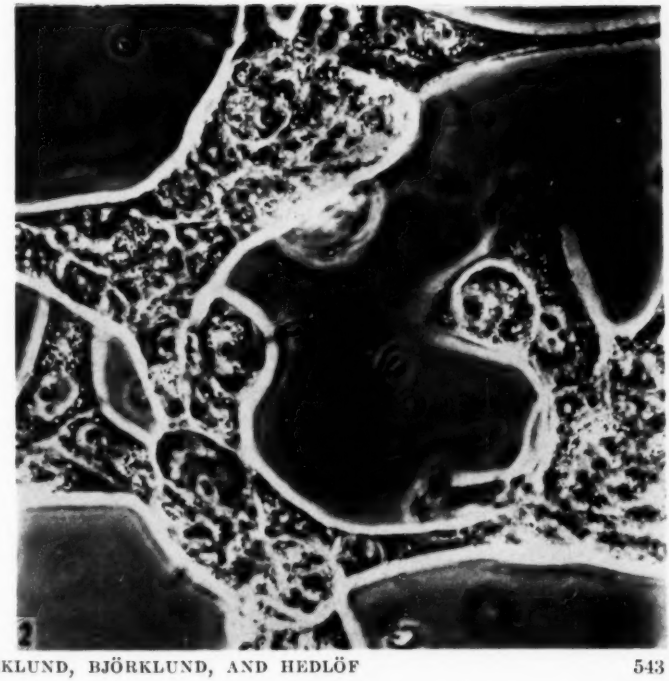
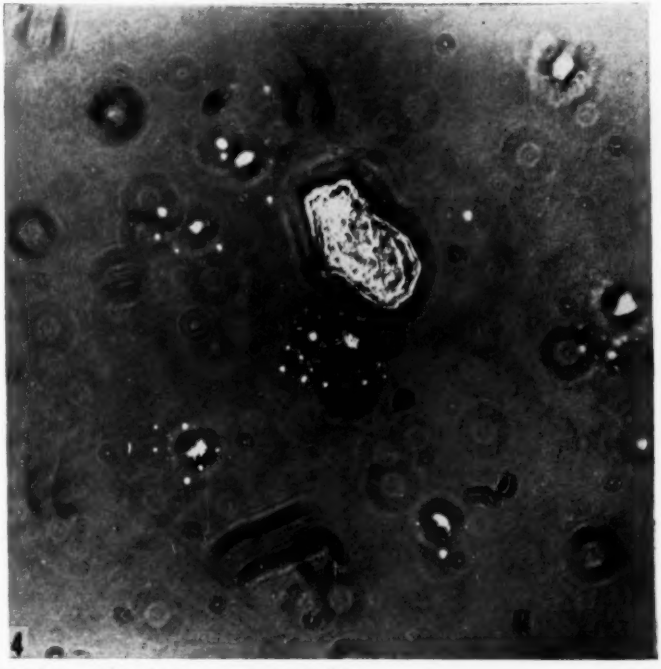


PLATE 77

FIGURE 3.—Complete clumping after exposure to horse antibodies diluted 1:40. This reaction, which is recorded as +++, represents a more advanced damage.

FIGURE 4.—Complete lysis or fragmentation of cells after exposure to antibody diluted 1:20.

3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The Antigens of Human Leukocytes. I. Introduction [1, 2]

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SUMMARY

Methods for the preparation of human leukocyte antigens for immunization and analysis by gel diffusion are presented. Contamination of these antigens by serum proteins was shown to be less than 2 percent. The properties of the antileukocyte serums are described.—J. Nat. Cancer Inst. 26: 547–555, 1961.

PREVIOUS IMMUNOLOGICAL studies of nucleated mammalian cells were performed usually by methods involving agglutination or complement fixation. These techniques cannot differentiate between the many cellular antigens against which antibody may be formed. Moreover, the cellular complexity of most tissues and organs raised some doubt whether the antigens studied were those of the tissue under consideration or of contaminating connective tissues.

The double-gel diffusion technique (*3*) overcomes the technical difficulties inherent in the study of complex immunological systems, because each antigen-antibody complex appears as a separate zone of precipitate; furthermore, two different tissues may be compared directly for their content of the same antigen. Leukocytes, especially from patients with leukemia, may be obtained in a relatively high degree of cellular homogeneity, which makes them suitable for immunological studies. This report will present the methods employed in the study of human leukocyte antigens.

MATERIALS AND METHODS

Leukocytes.—Normal leukocytes were obtained from pooled samples of heparinized normal blood, kindly provided by the blood bank of the

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[2] This investigation was supported by research grant C-2359 from the National Cancer Institute, National Institutes of Health, Public Health Service. Preliminary reports were presented at the meetings of the American Association for Cancer Research, 1957 and 1958 (*1, 2*).

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[4] The authors wish to acknowledge the technical assistance of Mr. Rudolph V. Smith.

580132—61——3

Sloan-Kettering Institute. Two volumes of blood were mixed with one volume of 3 percent polyvinylpyrrolidone (PVP) and kept in a siliconized cylinder at 37° C for 2 hours, at which time a sharp zone of demarcation between the erythrocytes and the plasma had been formed. The plasma, which contained the leukocytes, was centrifuged for 20 minutes at 3000 rpm. The resulting reddish sediment was subsequently suspended in hypotonic saline (0.57% NaCl), which caused the lysis of the erythrocytes without affecting the white cells. The leukocytes, removed by centrifugation and washed several times with hypotonic saline, formed a white suspension.

Leukemic leukocytes and those from persons with leukemoid reactions were isolated from 120 to 600 ml of blood obtained by exchange transfusion.

Preparation of leukocyte antigens.—The isolated leukocytes were kept in the deepfreeze at −20° C before preparation of the soluble antigens, and the frozen leukocytes were homogenized in cold physiological saline. The homogenate was centrifuged at 10,000 × *g* for 30 minutes at 0° C. The insoluble sediment, which was composed of cellular debris, was lyophilized and used for immunization; the supernatant was dialyzed against distilled water. The water-soluble fraction was separated from the water-insoluble fraction by centrifugation, and both were lyophilized and used as test antigens.

Preparation of tissue antigens.—Tissue antigens, including those of cells grown in tissue culture and human tumors obtained at surgery or from cortisonized rats, were prepared as previously described (*4*).

Antiserums.—Rabbits (2–3 kg) were inoculated intramuscularly once a week with 30 to 100 mg leukocyte sediment in Freund adjuvant (*4*). At least 2 animals were immunized with the same material whenever possible. Three weeks after the sixth injection the rabbits were bled and the antiserums tested for precipitating antibodies. If the antiserums were sufficiently strong, the rabbits were exsanguinated; otherwise, immunization was continued with usually 2 to 3 additional weekly injections followed by exsanguination. The gel diffusion technique of Ouchterlony has been described (*5*). All cups were filled twice with 0.2 ml of reagents. The antiserums were absorbed with pooled human plasma unless otherwise indicated and used either at full strength or occasionally at dilutions of either 1:2 or 1:3. The lyophilized antigens were dissolved in physiological saline for the immunological tests.

RESULTS

It was of interest to determine how many serum proteins could be detected in the leukocyte extracts. Consequently, leukocyte extracts were tested with antiserums against purified serum proteins (albumin, γ-globulin, β-lipoprotein, macroglobulin, siderophilin, and ceruloplasmin), as well as against plasma. The leukocyte extracts were tested in concentrations of 10 to 20 mg/ml. Most extracts contained some γ-globulin

and albumin, but, since the concentration of these proteins was less than 2 percent, it must be assumed that they were serum contaminants (fig. 1). However, with the antiserum against plasma, one serum protein could be demonstrated in such high concentration in the extracts of some leukocytes relative to its concentration in plasma that it may be a natural component of these cells (fig. 2). Comparison of this protein with various serum fractions obtained by zone electrophoresis (*6*) ascertained this to be a β-globulin (fig. 3), and subsequent experiments showed that it was neither siderophilin nor β-lipoprotein. Although the leukocytes had been thoroughly washed before extraction of the soluble antigens, it was nevertheless found that the remaining insoluble sediments contained enough adsorbed serum proteins to result in the occasional production of antibodies against them. For example, figure 4 shows the reactivity of one such antiserum with normal and leukemic plasmas.

Immunization with a mixture of antigens produces varying responses of antibody production in different rabbits, as shown in figure 5. Six different antiserums produced by injection of granulocytes from various sources contained antibody against several antigens. Most of the antiserums had antibody against the same granulocyte antigens, but antibody against several other antigens could be found only in a few of these antiserums; for example, *note* the difference between the two antiserums against normal leukocytes (cups D and E).

To avoid study of the distribution of the same antigen with two different antiserums and the assumption that two antiserums prepared against different cells react with different antigens, we made a comparative study. For example, figure 6 shows that antiserums produced against different cells may react with the same antigens, as indicated by coalescence of the lines. The two antiserums in figure 7 are obviously directed against different major antigens, since the lines intersect each other. Once a given antigen has been defined by an antiserum, its distribution in other cells can be studied.

Any study dealing with the antigenic composition of cells requires a reproducible method for the extraction of the antigens. Previous work with tissue antigens has shown that the methods we used were adequate (*5*), and the present study confirmed this finding (*see* "The Specificity of Leukocyte Antigens," p. 557).

Another problem which required attention was introduced by the fractionation of the leukocyte antigens in a water-soluble and water-insoluble fraction (*see* "Materials and Methods"). Since these fractions may differ in antigenic composition, it is essential to compare them with each other and to test both when the distribution of these antigens is studied for different cell types. The antigenic composition of the two fractions was indeed different (fig. 8). This fractionation was necessary because we found previously that the water-soluble fraction of granulocytes formed a strong precipitate with extracts of other cells or dialyzed normal serum or plasma (*7*). Since the plasma absorbing the antiserums was dialyzed before lyophilization, a strong interfering line will be pro-

duced between the cups containing the antiserums and the water-soluble granulocyte extract (fig. 8), and this line, which does not represent an immune system, may result in erroneous interpretations. Consequently, in studies requiring high antigen concentrations, water-insoluble fractions were used, for they do not contain the factor that is responsible for the nonimmune precipitins (*2*).

When an antiserum is produced against a mixture of antigens, the resulting antibodies may have different optimal zones of reactivity for the various antigens. In a complex immunological system it frequently happens that the concentration of one antigen will be such that it will form a line, whereas the concentration of a second antigen is unsuitable for the formation of a precipitate. As the optimal conditions of the various immune systems may differ, no given concentration of the leukocyte extract will produce the possible maximum number of lines. Therefore, such a mixture must be titrated and studied in the concentration range in which the important antigens manifest themselves. Such a titration of a leukocyte extract is illustrated in figure 9, which also indicates the lowest concentration at which any antigen still can be detected in the homologous leukocyte extract, data that are essential for the future study of the relative specificities of these antigens in other cells. The serial dilution of the leukocyte extract does not affect all antigens in the same manner. For example, some of the lines formed by slowly diffusing antigens, as indicated by the curvature of the line (fig. 8) away from the antibody cup, disappear after as little as a fourfold dilution, whereas others are still strong at dilutions of 0.5 mg/ml. Some of the antigens are detectable only at concentrations of 20 mg/ml dry weight, which sets a lower limit to the amount of leukocyte extract required for extensive immunological studies.

During the last 3 years many rabbits were immunized with leukocytes from patients with different forms of leukemia. Although individual rabbits varied widely in their response to the same antigens, it is nevertheless our impression that some leukocytes are better immunizing antigens than others. Good antiserums were invariably obtained against normal granulocytes and granulocytes from patients with a leukemoid reaction or chronic myelogenous leukemia; those from chronic lymphocytic leukemia were the poorest immunizers.

Immunization with the latter types frequently led to the formation of antibody against an antigen which was present in very low concentration in the homologous cells. This antigen was presumably contributed by the contaminating granulocytes, which contain large amounts of it. Examples will be given in "The Specificity of Leukocyte Antigens," page 557.

DISCUSSION

The value of the double-gel diffusion method for the study of complex antigenic systems was apparent early. Björklund (*9*) showed that horse antiserums against rabbit bone marrow gave rise to several lines with tissue extracts, and we have since then shown that by this method system-

atic studies can be made of human tissue antigens and some of their properties (*4*, *5*). The first report dealing with the immunological analysis of human leukocytes by the double-gel diffusion method appeared in 1955 (*10*).

We realized from our study of tissue antigens that it would be difficult to wash away all the absorbed serum proteins from the cells to be used for immunization, and prolonged washing of the cells could remove many of the smaller cellular antigens. Therefore, this treatment was limited to a minimum, and the antiserums were absorbed with lyophilized human plasma to remove antibody against plasma proteins. Although we did not attempt to wash away the last traces of serum proteins, their concentration in the leukocyte extracts was quite low. For example, serum albumin, which is the most concentrated protein of serum, comprised less than 2 percent of the leukocyte extract. The serum proteins could be detected because large amounts of the leukocyte extract were tested rather than the wash solutions (*11*).

Albumin was present in low concentrations in the leukocyte extracts; however, greater amounts of ceruloplasmin or macroglobulin in the extracts of leukemic lymphocytes had been expected since the serums from leukemic patients often contain increased amounts of ceruloplasmin (*12*), and macroglobulinemia is usually associated with increased numbers of "lymphocyte-like" cells (*13*). Moreover, many patients, who are clinically indistinguishable from those with chronic lymphocytic leukemia or lymphosarcoma, frequently have macroglobulinemia (*14*).

A β-globulin found in normal human serum was present in relatively high concentration in the extracts of some leukocytes, and it seems that these cells are involved in the synthesis of this protein.

The difficulties inherent in all studies involving tissue antigens became apparent in this work. The most serious difficulty is, of course, the dependence of the investigator on the antibody-producing animal. Variations among different rabbits inoculated with the same tissue extracts limit the work to a given antiserum, since it is unlikely that a similar one may be produced again. Moreover, some of the antigens, even when present as trace contaminants, can be potent antibody inducers resulting in antiserums against antigens, which may not be of great interest in a study of antigenic differences among various cell types. Another difficulty is presented by the quantitative limitations of the double-gel diffusion method, since some antigens are present in amounts too small to be detected by the antiserums unless the antigenic extracts are used at very high concentrations. This limitation becomes even more pronounced when potent antiserums are used, because it is one of the paradoxes of the double-gel diffusion method that the more potent the antiserum the less of a given antigen it can detect.

This study was concerned only with those antigens which are extracted from the cells and which diffuse through the agar. In the following paper we will show that it is possible to check the antigens of the insoluble cellular residue by suitable absorption procedures.

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PLATE

PLATE 78

Abbreviations for legends: NWBC = normal white blood cells; LR = leukemoid reaction; CML = chronic myelocytic leukemia; AL = acute leukemia; CLL = chronic lymphocytic leukemia. Numbers following abbreviations refer to the patient: *i.e.*, CLL 22: antigen extract of cells from patient #22 with chronic lymphocytic leukemia; → = evolving into.

FIGURE 1.—Center cup: antialbumin serum; A: CML→AL 8, 20 mg/ml; B: CLL 18, 20 mg/ml; C: CLL 14, 20 mg/ml; D: Crystalline mercaptalbumin, 0.4 mg/ml; E: CLL 22, 20 mg/ml; F: AL 33, 20 mg/ml.

FIGURE 2.—Center cup: antihuman plasma serum; A and D plasma (1:5); B: CLL 21, 20 mg/ml; C: CLL 18, 20 mg/ml; E: CML→AL 8, 20 mg/ml; F: CLL 22, 20 mg/ml.

FIGURE 3.—Center cup: antihuman plasma serum; A: CML 8, 20 mg/ml; B and D: β-globulin, 10 mg/ml; C: AL 10, 20 mg/ml; E: CLL 18, 20 mg/ml; F: α_2-globulin, 10 mg/ml.

FIGURE 4.—Center cup: anti-AL 4, serum not absorbed; A: AL 4, plasma; B: CML 1, plasma; C: CLL 6, plasma; D: CLL 3, plasma; E: AL 5, plasma; F: normal, plasma. All plasma samples were used at a twofold dilution.

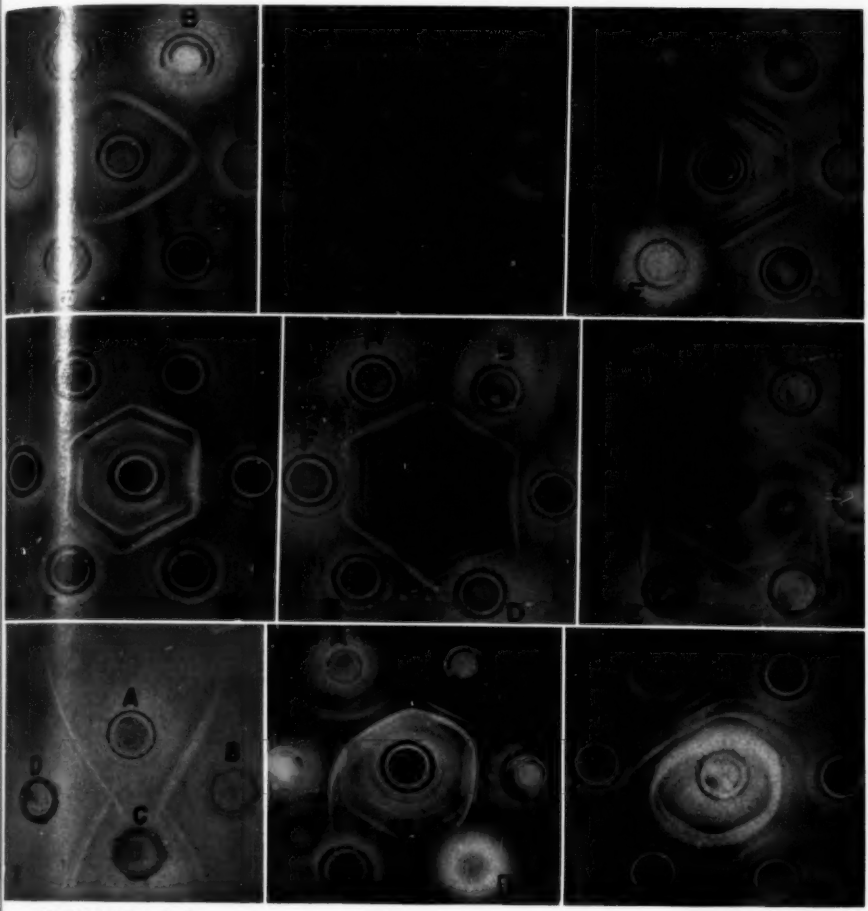
FIGURE 5.—Center cup: CML 13, 4 mg/ml; A: anti-CML 1, serum; B: anti-CML 9, serum; C: anti-CML 13, serum; D: anti-NWBC; A, serum; E: anti-NWBC; B, serum; F: anti-LR, serum.

FIGURE 6.—Center cup: mixture of AL 4 and CML 24; A: anti-AL 4, serum; B: anti-CML 13, serum; C: anti-CLL 11, serum; D: anti-AL 10, serum; E: anti-CML, 8, serum; F: anti-CML 1, serum (1:3).

FIGURE 7.—A: anti-CML, serum (1:3); B: CML 12, 5 mg/ml; C: anti-NWBC A, serum; D: CML 1, 5 mg/ml.

FIGURE 8.—Center cup: anti-CML 1, serum (1:3); A: CML 9; B: CML 9, water insoluble; C: CLL 3, water insoluble; D: CLL 3; E: AL 10; F: AL 10, water insoluble. All antigens were used at 10 mg/ml.

FIGURE 9.—Center cup: anti-CML 13; A: CML 13, 20 mg/ml; B: CML 13, 10 mg/ml; C: CML 13, 5 mg/ml; D: CML 13, 2.5 mg/ml; E: CML 13, 10 mg/ml; F: CML 13, 0.5 mg/ml.



580122—61——4

The Antigens of Human Leukocytes. II. The Specificity of Leukocyte Antigens [1, 2]

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SUMMARY

Antiserums were prepared against normal and leukemic granulocytes, against the white cells of acute leukemic patients, and against lymphocytes from patients with lymphocytic leukemia. With these antiserums several leukocyte antigens could be identified, and their distribution in various cells and organs was investigated. One of these antigens was highly specific for granulocytes; another could be found only in the leukocytes from certain patients with acute leukemia, as well as in several normal organ extracts. No antigen specific for the lymphocytes was found, but the lymphocytes from different patients varied in their content of several antigens. As yet it has not been possible to detect antigenic differences between normal granulocytes and those from patients with chronic myelogenous leukemia.—J. Nat. Cancer Inst. 26: 557–567, 1961.

IN THE preceding paper (*1*) it was shown that potent precipitating antiserums can be produced against soluble human leukocyte antigens. This report will present data on the distribution of several of these antigens in different cells and tissues.

MATERIALS AND METHODS

Leukocytes.—The methods used for the isolation of normal leukocytes and those from patients have been described (*1*), as were the methods for the preparation of the leukocyte antigens. The 39 patients whose leukocytes were studied had the following hematological abnormalities:

Leukemoid reaction	2
Acute leukemia	14
Chronic lymphocytic leukemia	13
Chronic myelocytic leukemia	10

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[4] The authors wish to acknowledge the technical assistance of Mr. Rudolph V. Smith.

580132—61——5

Data regarding the blood types of most of the patients are available as are their differential and total counts; however, because of lack of correlation between the antigenic analysis and the distribution of the various cell types in any given sample, no tabulation of the differential counts is presented, nor will the acute leukemias be further divided on a morphological basis.

The terms "granulocyte" and "lymphocyte" are used for convenience in referring to cells from the blood of patients with chronic myelocytic leukemia and chronic lymphocytic leukemia, with the appreciation that the leukemia may not present a completely homogeneous leukocyte population.

Human tumor cells, grown either in cortisonized animals (*2*) or in tissue culture, were kindly supplied by Dr. H. Toolan and Dr. C. Southam of the Sloan-Kettering Institute for Cancer Research. These cells were: HeLa cells; a melanoma; four epidermoid carcinomas, HEp #1, HEp #2, HEp #3, and HEp #5; a sarcoma, HS #1 (*2*), and two cell lines isolated from the buffy coats of the blood of patients with monocytic leukemia, Osgood J111, and Osgood J96 (*3*). Surgical or autopsy specimens of normal organs or tumors were obtained from Memorial Hospital. The extraction of soluble antigens from these cells and tissues has been described (*4*).

The methods used for analysis by gel diffusion and the techniques employed for the preparation of the antiserums used in this study have been described in the preceding paper (*1*).

RESULTS

The first cell system which was studied extensively was that of the granulocyte. Potent antiserums could be produced regularly against these cells. It soon became apparent that one of the antigens reacting with these antiserums could be detected when extracts of granulocytes were used even at very low concentration. This antigen (#1), however, was present in very small amounts, if at all, in other cell types. For example, in figure 1 extracts of 5 different cell types were compared. The homologous system reacted strongly at an antigen concentration of 1 mg/ml (cup D), and it was still reactive at one tenth this concentration. The other cell types, which included 2 chronic lymphocytic leukemias at B and C, and 1 acute leukemia, did not react at concentrations of 5 mg/ml. A second chronic myelogenous leukemic extract at F reacted strongly under these conditions. However, if extracts from the cells that did not react were tested at higher concentrations (for example at 20 mg/ml), antigen #1 could be detected. This raised the question whether cells other than the granulocytes produced this antigen, though in very small amounts, or whether its presence was due to the few contaminating granulocytes. None of the antiserums could distinguish

between normal leukocytes and those of patients with chronic myelogenous leukemia or leukemoid reaction.

A second antigen (#2), which seemed to occur in relatively high concentration in granulocytes but at much lower concentrations in other cell types, was detected with an antiserum against normal leukocytes (fig. 2); the differential, however, was not as great as with the previous antigen. Interesting results were obtained when the water-insoluble (but saline-soluble) fractions of leukocytes were tested at high concentrations with this antiserum (figs. 3 and 4). It may seem strange that the homologous system (fig. 3, cup A) contains fewer antigens than do the extracts of cells from patients with chronic myelogenous leukemia. This, however, is a function of the concentration of the antigen and the homogeneity of the cellular population; since the normal white-cell population is composed of both granulocytes (approximately 65–70%) and lymphocytes, it is not surprising that the concentration of a granulocyte antigen should be lower in the extract of normal leukocytes than in the extract of the more homogeneous leukemic cells. The low concentration of many of the antigens in cells other than granulocytes is apparent from figure 4. Similar results were obtained with another antiserum against granulocytes from a patient with chronic myelogenous leukemia (fig. 5).

Are the antigens of the leukocytes also present in other cells? An answer to this question is not easy, because human organs and tissues invariably contain contaminating cells. The availability of human cells grown in cortisonized rats or tissue culture avoids this complication, but the possibility that such cells may have lost some of their antigens must be considered. From our studies it became apparent that some of the antigens found in leukocytes were also shared by other cells. For example, figures 6 and 7 show that 4 cell types grown in tissue culture or in cortisonized rats produce varying amounts of antigens capable of reacting with the antileukocyte serums. It is of interest that 2 of these cell types, the Osgood J111 (figs. 6F and 7F) and J96 were isolated from the buffy coat of the blood from patients with acute monocytic leukemia. On the other hand, some antigens that are present in granulocytes could not be detected in extracts of cells grown in tissue culture, even at concentrations of 40 mg/ml of a pure HeLa culture (fig. 7, cup E). These antigens are therefore produced in large amounts by granulocytes but not at all, or in very small amounts only, by other cells grown in tissue culture or in cortisonized rats (*see also* fig. 4, cup B). Extracts of surgical specimens of tumors and tissues were also compared with the leukocytes and large quantitative differences in their antigenic content were observed. Figure 8 shows that a human reticulum-cell-sarcoma extract at 40 mg/ml (cup B) and the extract of normal uterine tissue (cup C) are lacking in at least one antigen that is detectable in 2.5 mg/ml of the homologous extract. The same seems to be true for a human sarcoma (fig. 9, C); HEp #2, grown in tissue culture (cup B), has only one antigen in common with the granulocytes. The antigenic difference between a lymph-node extract at 40 mg/ml (cup F) and the granulocytes is of

interest. The nature of the lines between the cups containing the granulocyte extracts and the cups containing extracts from other cells has been described previously (*1*).

It can therefore be said that human granulocytes contain large amounts of several antigens that are either not produced at all by other cells or produced only in such small amounts that they cannot be detected by our methods. However, other granulocytic antigens are shared by all other cells tested.

The second group of cells characterized by a relatively specific antigen (#3) was obtained from some patients with acute leukemia. Antiserums against such cells from one patient (anti-AL 4, serum) produced a distinct line with the homologous cell extract and the extract of certain other acute leukemic cells but not with extracts of other leukocytes at comparable concentration ranges (figs. 10 and 11).

This antigen could be detected in concentrations as low as 2.5 mg/ml of the homologous extract. In extracts from cells of 2 patients there was, in addition to antigen #3, a strong line in common with the granulocyte extracts (fig. 11, cup F). Both of these patients were diagnosed as having subacute myelogenous leukemia, which probably represents a transition from chronic to acute myelogenous leukemia.

Antigen #3 was absent from human cells grown in cortisonized rats as well as from the Osgood J111 and J96. Nevertheless, this antigen was not specific for the leukocytes from acute leukemia patients since it was present also in normal liver, lymph nodes, and normal spleen (fig. 12).

Anti-AL 4 serum reacts also strongly with an antigen present in granulocyte extracts, but hardly at all in the homologous extract. This phenomenon, which has already been described in the first paper of this study (*1*), must be due to the high antigenicity of this antigen, which was probably contributed by the few granulocytes that were present in the preparation used for immunization. This antigen also occurs in concentrated normal plasma (fig. 10, cup D). [The splitting of the line is a common finding for systems in antibody excess (*5*).] That leukocyte antigens can be detected in normal serums has already been shown by Seligmann (*6*).

Antiserums prepared against cells from other patients with acute leukemia failed to detect antigen #3, nor did they react with a specific homologous antigen. Cells from patients with lymphocytic leukemia contained only small amounts of the antigens that could be detected with these antiserums (fig. 13).

Although lymphocytes are relatively weak immunizing antigens (*1*), several strong antilymphocyte antiserums were obtained. Nevertheless, no antigens specific for the lymphocyte could be demonstrated with them. Many of the antigens reacting with these antiserums were present in much higher concentration in the granulocytes than in the lymphocytes, which suggests that the antibodies were produced in response to contaminating granulocyte antigens (fig. 14). The antigenic variations among different lymphocytes were an unexpected finding (fig. 15). In this instance the

homologous lymphocytes contained much less of some antigens than did the other lymphocytes.

Absorption Experiments

It was suggested (*1*) that some antigens might be so tightly bound to the cellular debris of certain cells that they could not be extracted. To check this possibility, as well as to obtain more specific antiserums without increasing their protein concentration, we absorbed a few antiserums with the saline-insoluble residues of homologous and heterologous cells. Different amounts of insoluble residue were used for the absorptions. Absorption of 1 ml of antinormal white cell serum with 20 or 40 mg residue of lymphocytic leukemic cells did not remove much antibody against one of the antigens (fig. 16), whereas absorption with 20 mg of a chronic myelogenous leukocyte residue removed all antibody. Similar results were obtained with an antiserum against chronic myelogenous leukemic cells. Figure 17 shows the pattern obtained before absorption with 40 mg/ml of a lymphocytic residue, and figure 18 shows the pattern after absorption. These experiments indicate that a low concentration or absence of certain leukocyte antigens from lymphocytic cells cannot be explained on the basis of differences in extractibility, and that antiserums may be prepared which will no longer react with lymphocytes.

DISCUSSION

Our data show that white cells can be differentiated on the basis of their content of certain antigens. At present we can distinguish three groups:

Group I.—This group is composed of normal leukocytes and cells from patients with chronic myelogenous leukemia and from patients with leukemoid reaction. These leukocytes, which are characterized by a predominance of granulocytes, contain considerable amounts of antigens #1 and #2 and little of antigen #3.

Group II.—This group of leukocytes contains cells from certain acute leukemia patients and are not further characterized morphologically. These relatively immature cells are rather deficient in antigen #1 but contain considerable amounts of antigen #3, which, although present in some acute leukemic cells, is absent from others and from lymphocytes and mature granulocytes. It is not specific for immature leukocytes, however, since it has been found in normal spleen and liver.

Group III.—This group is composed of cells that are relatively deficient in most of the antigens found in high concentration in cells of groups I and II and includes certain cells of the acute leukemias and all cells from patients with chronic lymphocytic leukemia. The two leukocyte cell lines grown in tissue culture, *i.e.*, the Osgood J111 and J96, also belong to this group.

The fact that members of the granulocytic series contain large amounts of certain antigens that are lacking, or present in low concentration only,

in other leukocytes is not surprising, since the granulocytes contain, besides their granules, enzymes which so far have not been demonstrated in other leukocytes—for example, myeloperoxidase (*7*). We have as yet no explanation for the distribution of antigen #3 which occurs in certain cells of the acute leukemias and normal organs. However, the presence of this antigen may be related to the degree of maturity of these cells.

The fact that by proper absorption antiserums specific for granulocytes may be obtained suggests that these antiserums, when labeled with fluorescein, can be used for immunohistological studies.

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PLATES

PLATE 79

Abbreviations for legends: NWBC = normal white blood cells; LR = leukemoid reaction; CML = chronic myelocytic leukemia; AL = acute leukemia; CLL = chronic lymphocytic leukemia; TC = grown in tissue culture; CR = grown in cortisonized rats. Numbers following the abbreviations refer to the patient: *i.e.*, CLL 22: antigen extract of cells from patient #22 with chronic lymphocytic leukemia; → = evolving into.

FIGURE 1.—Center cup: anti-CML serum; A: CML 1, 2.5 mg/ml; B: CLL 17, 5.0 mg/ml; C: CLL 18, 5.0 mg/ml; D: CML 1, 1.0 mg/ml; E: AL 19, 5.0 mg/ml; F: CML 13, 5.0 mg/ml.

FIGURE 2.—Center cup: anti-NWBC serum; A: CML 9, 5 mg/ml; B: CLL 18, 5 mg/ml; C: CLL 21, 5 mg/ml; D: CML 24, 5 mg/ml; E: CLL 22, 5 mg/ml; F: AL 23, 5 mg/ml.

FIGURE 3.—Center cup: anti-NWBC serum; Cups A to F water-insoluble fractions of: A: NWBC, 10 mg/ml; B: CML→AL 8, 10 mg/ml; C: AL 2, 10 mg/ml; D: CML 24, 10 mg/ml; E: CML 13, 10 mg/ml; F: CLL 3, 10 mg/ml.

FIGURE 4.—Center cup: anti-NWBC serum; A: CML 13, 10 mg/ml; B: HEp #2 (TC), 20 mg/ml; C: CLL 21, 10 mg/ml; D: CML 24, 10 mg/ml; E: CLL 22, 10 mg/ml; F: CLL 14, 10 mg/ml.

FIGURE 5.—Center cup: anti-CML 24A, serum; Cups A to F water-insoluble fractions of: A, D: CML 24, 20 mg/ml; B: CML 13, 20 mg/ml; C: CML 12, 20 mg/ml; E: CML→AL 8, 20 mg/ml; F: CLL 18, 20 mg/ml.

FIGURE 6.—Center cup: anti-NWBC serum; A, D: CML 1, 2.5 mg/ml; B: HEp #3 (CR), 40 mg/ml; C: HEp #5 (CR), 40 mg/ml; E: HS #1 (CR), 40 mg/ml; F: Osgood J111 (TC), 30 mg/ml.

FIGURE 7.—Center cup: anti-NWBC serum; A, D: CML 9, 2.5 mg/ml; B: HEp #2 (TC), 30 mg/ml; C: Melanoma (CR), 40 mg/ml; E: HeLa (TC), 40 mg/ml; F: Osgood J111 (TC), 20 mg/ml.

FIGURE 8.—Center cup: anti-CML 24B, serum; A: CML 24, 10 mg/ml; B: Reticulum-cell sarcoma, 40 mg/ml; C: Normal uterus, 40 mg/ml; D: CML 24, 2.5 mg/ml; E: Mammary carcinoma, 40 mg/ml; F: Normal spleen, 20 mg/ml.

FIGURE 9.—Center cup: anti-CML 24B, serum; A: CML 24, 10 mg/ml; B: HEp #2 (TC), 30 mg/ml; C: sarcoma, 40 mg/ml; D: CML 24, 10 mg/ml; E: carcinoma, 40 mg/ml; F: lymph node, 40 mg/ml.

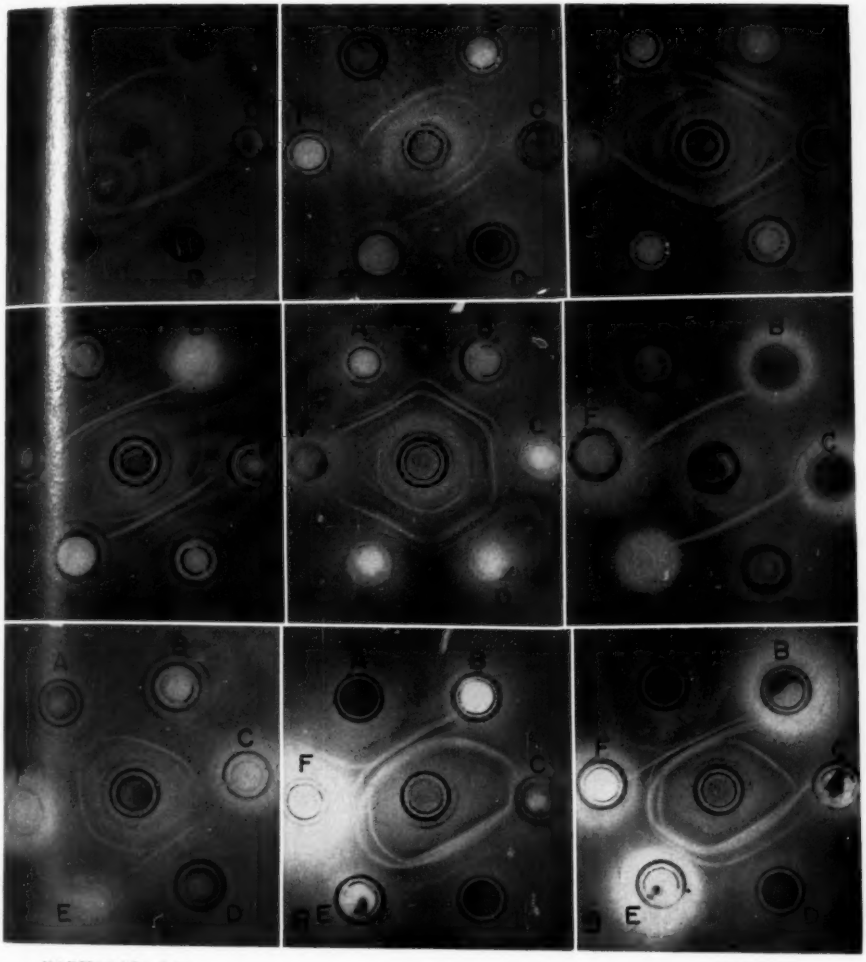
A
B
C
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PLATE 80

FIGURE 10.—Center cup: anti-AL 4, serum; A: AL 4, 10 mg/ml; B: CLL 3, 10 mg/
C: CML 1, 10 mg/ml; D: normal plasma, undiluted.

FIGURE 11.—Center cup: anti-AL 4, serum; A: AL 4, 5 mg/ml; B: NWBC, 5 mg/
C: CML 1, 2.0 mg/ml; D: AL 2, 5.0 mg/ml; E: CML 9, 5.0 mg/ml; F: AL 7,
mg/ml.

FIGURE 12.—Center cup: anti-AL 4, serum; A, D: AL 10, 20 mg/ml; B: CLL
20 mg/ml; C: CLL 22, 20 mg/ml; E: CLL 26, 20 mg/ml; F: CLL 21, 20 mg/ml.

FIGURE 13.—Center cup: anti-AL 10, serum; A, D: AL 10, 20 mg/ml; B: CLL
20 mg/ml; C: CLL 22, 20 mg/ml; E: CLL 26, 20 mg/ml; F: CLL 21, 20 mg/ml

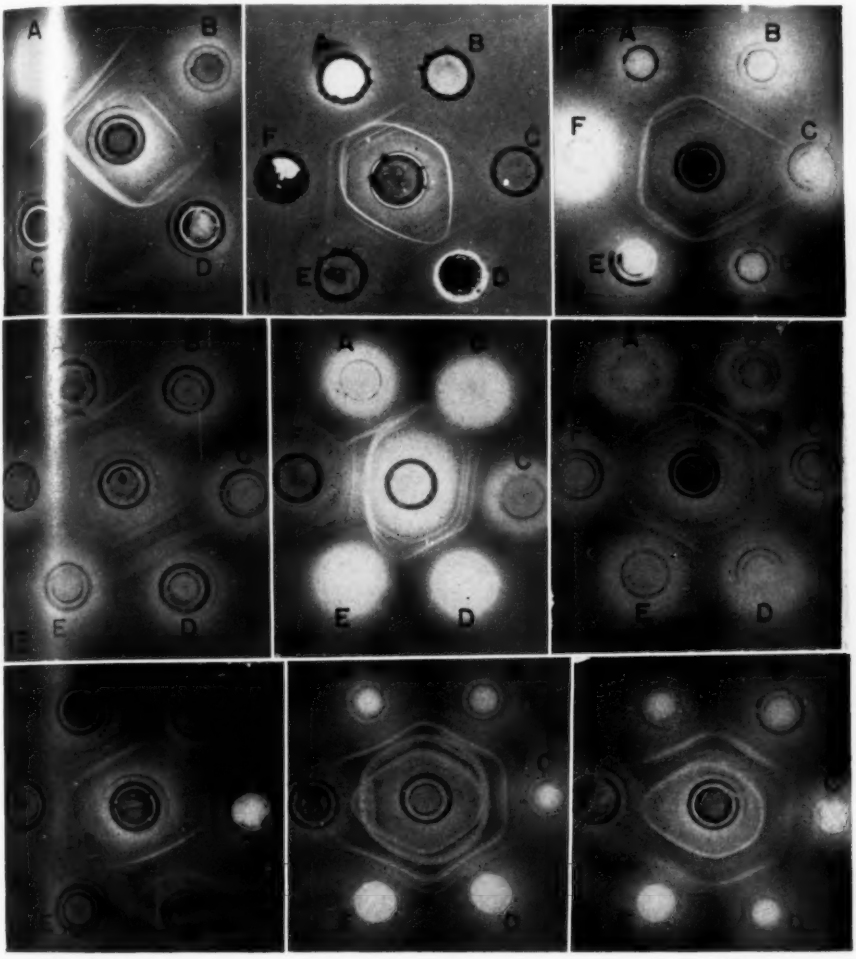
FIGURE 14.—Center cup: anti-CLL 11, serum; A, D: CLL 11, 20 mg/ml; B: C
22, 20 mg/ml; C: CLL 17, 20 mg/ml; E: CLL 18, 20 mg/ml; F: CML 24, 20 mg/

FIGURE 15.—Center cup: anti-CLL 18, serum; A, D: CLL 18, 20 mg/ml; B: CLL
20 mg/ml; C: CLL 21, 20 mg/ml; E: CLL 26, 20 mg/ml; F: CLL 22, 20 mg/ml.

FIGURE 16.—Center cup: anti-NWBC serum absorbed with 40 mg/ml of CLL residu
A: CML 13, 5 mg/ml; B: CLL 21, 5 mg/ml; C: AL 2, 5 mg/ml; D: CML 9, 5
mg/ml; E: CML 12, 5 mg/ml; F: CLL 3, 5 mg/ml.

FIGURE 17.—Center cup: anti-CML 24B, serum; A to F, water-insoluble fractions of:
A, D: CML 24, 20 mg/ml; B: CML 13, 20 mg/ml; C: CML 12, 20 mg/ml; E:
CML 8→AL 8, 20 mg/ml; F: CLL 18, 20 mg/ml.

FIGURE 18.—Center cup: anti-CML 24B, serum absorbed with 40 mg/ml CLL
residue. A to F as in figure 17.



589132—61——6

Homologous and Heterologous Skin Transplantation in Patients With Lymphomatous Disease [1]

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SUMMARY

Forty-three patients with lymphomatous disease received homologous skin transplants. In 19, survival of the graft was prolonged. Nine patients who had received human skin grafts also received pigskin transplants. In 2, survival was prolonged. There was no consistent correlation between ability to accept or reject homografts and type of therapy administered, the titer of isohemagglutinins, response to typhoid-paratyphoid vaccination, skin testing with tuberculin, mumps, and *Candida albicans* antigens or properdin titers. Graft survival was not prolonged in the 4 patients with hypogammaglobulinemia. There were patients with chronic lymphatic leukemia, Hodgkin's disease, lymphosarcoma, reticulum-cell sarcoma, and multiple myeloma in both the typical-rejection and prolonged-survival groups. Two patients with chronic myelogenous leukemia and 1 with acute leukemia had typical homograft rejections.—J. Nat. Cancer Inst. 26: 569–583, 1961.

PROLONGED SURVIVAL of skin homografts in patients with lymphomatous disease has been reported (*1, 2*). Patients with these diseases have also shown a deficiency in immune responses (*3, 4*). In addition, these diseases may be associated with acquired agammaglobulinemia, a condition in which successful homografting has occurred (*5*). It is the purpose of this paper to report the results of skin homografting in 43 patients with the following diseases: Hodgkin's disease, lymphosarcoma, reticulum-cell sarcoma, chronic lymphatic leukemia, chronic myelogenous leukemia, acute leukemia, and multiple myeloma. The results of homografting are correlated with those of the determination of serum electrophoresis, titer of isohemagglutinins, response to typhoid-paratyphoid vaccination, titer of properdin, and response to skin testing with tuberculin, mumps, and *Candida albicans* antigens. As further evaluation of the degree to which the immune barrier had been breached by the lymphomatous disease, 9 of these patients received transplants of heterologous skin.

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580132—61——7

MATERIALS AND METHODS

Forty-one patients received adult skin homografts; in 2, the skin was fetal in origin. The surgical technique used for the adult and fetal donor and the recipient and the criteria for evaluation of survival or rejection of the homograft have been described (*6*).

Preparation of heterologous skin.—Suckling pigs less than 1 week old were killed by ether anesthesia. The thin skin of the pectoral and inguinal regions was removed aseptically and grossly freed of subcutaneous fat by dissection. The remainder of the procedure was the same as that described for homologous skin (*6*).

Serum electrophoresis.—For each specimen, the determination was made in duplicate on an 8-strip Spinco Model R paper electrophoresis cell with the use of 3 cm paper strips (Whatman #308–028). The stained strips were analyzed with a calibrated recording photometer and automatic integrator and expressed in grams percent of protein.

Isohemagglutinins.—The serum to be tested for isohemagglutinins was diluted in saline by standard serial dilution methods. Washed red cells were suspended in saline to make a 2 to 3 percent preparation. An equal volume of appropriate suspended cells was added to each tube and allowed to incubate at room temperature for 1 hour. The mixture was then centrifuged, agitated, and examined grossly for agglutination. The tube after the last tube to show gross agglutination was examined microscopically and this procedure was continued until no agglutination was seen.

Response to typhoid-paratyphoid A and B vaccination.—The typhoid and paratyphoid vaccine contained, per cm^3, one billion killed typhoid bacilli, 250 million killed paratyphoid A, and 250 million killed paratyphoid B bacilli. Each patient received 3 subcutaneous injections of 0.5 cc. of vaccine at least 1 week apart. If more than 1 month elapsed between injections, the entire series was repeated. Blood for antibody response was taken 3 to 4 weeks after the last vaccination. Serum specimens to be tested for agglutinating antibodies were set up with 4 antigens, with a microscopic slide method. The antigens were B. Typhosis O, B. Typhosis H, B. Paratyphosis A, and B. Paratyphosis B. Antigens used for the diagnostic slide agglutination were prepared according to the method of Welch and Stuart (*7*).

Tuberculin, mumps, and Candida albicans *skin tests.*—Tuberculin-purified protein derivative was given in a dose of 2×10^{-7} g per each 0.1 cc injection (intermediate strength). Freshly prepared mixtures were used for all tests. The skin-test antigen for mumps-virus hypersensitivity was prepared with ultraviolet-light-inactivated virus grown in embryonated chicken eggs. Each cc of skin-test antigen contained 20 complement-fixing units. *C. albicans* skin tests were performed with a 1:1000 dilution of mold extract (provided by the courtesy of the Hollister-Stier Laboratories, Philadelphia, Penna.). In each case, 0.1 cc of the skin-test antigen solution was injected intradermally into the volar surface of the forearm with at least 2 inches between injection sites. All tests were read in

48 hours. A positive reaction was recorded for the tuberculin and *C. albicans* skin tests when the injection site showed a palpable area of induration at least 6 mm in diameter with or without redness. A positive mumps skin test required an area of erythema at least 1.5 cm in diameter with or without induration.

Properdin titer.—Properdin was determined by zymosan (hemolytic) assay as described by Pillemer *et al.* (*8*).

RESULTS

Table 1 presents the data on diagnosis, treatment, and number of days the grafts survived, and the serum electrophoretic findings in the patients who were separated into 2 groups. The Typical Rejection group all showed evidence of graft rejection before 30 days. The Prolonged Survival group retained the homograft for more than 30 days without evidence of rejection. All treatment given the patients within 30 days before or after transplantation was recorded. In the Typical Rejection group were 9 patients with chronic lymphatic leukemia, 6 with Hodgkin's disease, 4 with multiple myeloma, 2 with chronic myelogenous leukemia, 1 with acute leukemia, 1 with reticulum-cell sarcoma, and 1 with lymphosarcoma. In the Prolonged Survival group were 7 patients with chronic lymphatic leukemia, 5 with Hodgkin's disease, 3 with reticulum-cell sarcoma, 2 with lymphosarcoma, and 2 with multiple myeloma. Treatment with adrenocortical steroids, alkylating agents (adrenocortical steroids refer to cortisone, hydrocortisone, prednisone, triamcinolone, and dexamethasone; alkylating agents refer to nitrogen mustard, chlorambucil, diepoxypropylpiperazine, and epoxypropidine), and radiation therapy was administered to patients in both groups. Two patients in the Prolonged Survival group were treated with a combination of agents not represented in the Typical Rejection group. Both patients had multiple myeloma. H. Hen. received prednisone, radiation therapy, and urethan. M. O. received 6-mercaptopurine and 6-diazo-5-oxo-norleucine. The graft in patient M. O. was fetal in origin. The long-surviving homograft in patient M. I. (reticulum-cell sarcoma) treated with adrenal steroids and epoxypiperazine was also fetal in origin. Five patients in each group received no therapy for their disease. Of the 19 patients in the Prolonged Survival group, evidence of graft rejection after 30 days was seen in 3 patients. In 10 patients the graft was still viable at the time of this report; 3 patients died with the graft intact and in 3 patients there was inadequate followup.

Serum electrophoretic values for the 2 groups of patients are presented in table 1. Hypogammaglobulinemia occurred in 4 patients in the Typical Rejection group. There was no hypogammaglobulinemia in the Prolonged Survival group. Two patients in the Typical Rejection group had hypergammaglobulinemia. This was found in 3 patients who retained their homografts for unusual periods. A moderate elevation of α- and

TABLE 1.—Diagnosis, therapy, and serum electrophoretic findings in patients showing typical rejection and prolonged survival of homografts

Patient	Diagnosis	Therapy within 30 days of homografting	Homograft survival (days)*	Serum electrophoresis values					
				Total	Albumin	Alpha-1	Alpha-2	Beta	Gamma
	A. Typical rejection								
J. D.	Chronic lymphatic leukemia (CLL)	Adrenal steroid†	14	6. 3	4. 20	0. 31	0. 60	0. 88	0. 31
H. Her.	"	" "	17	7. 5	4. 84	0. 41	0. 85	0. 78	0. 65
N. H.	"	" "	10	7. 1	4. 60	0. 35	0. 72	0. 92	0. 47
S. L.	"	" "	7	5. 9	2. 93	0. 26	1. 02	1. 07	0. 62
B. M.	"	None	14	5. 4	3. 00	0. 32	1. 00	0. 94	0. 16
C. Ri	"	Adrenal steroid	<30	6. 7	3. 51	0. 31	0. 74	1. 04	1. 11
C. S.	"	None	14	7. 2	3. 90	0. 40	1. 08	0. 91	0. 91
N. S.	"	"	14	8. 4	4. 60	0. 37	0. 50	1. 35	1. 57
J. T.	"	Adrenal steroid	27	5. 7	3. 75	0. 26	0. 46	0. 75	0. 49
W. Co.	Hodgkin's disease (HD)	" " X ray	19	5. 9	2. 04	0. 41	0. 72	1. 26	1. 47
F. G.	"	X ray, alkylating agent‡	28	—	—	—	—	—	—
H. M.	"	X ray	14	7. 4	3. 98	0. 27	0. 45	1. 18	1. 52
C. Ra.	"	Adr. st., alkylating agent	<30	5. 4	2. 04	0. 51	1. 21	0. 97	0. 68
				NORMAL RANGE					
G. S.	"	X ray	19						
G. W.	"	Alkylating agent	27	6. 5—	3. 75—	0. 13—	0. 31—	0. 48—	0. 63—
C. M.	Chronic myelogenous leukemia (CML)	None	<30	7. 9	5. 23	0. 29	0. 89	1. 06	1. 77
A. Y.	"	Antimetabolite§	<30	6. 4	2. 77	0. 35	0. 81	1. 03	1. 45
H. Hei.	Lymphosarcoma (LSA)	Adr. st., X ray	7	6. 5	1. 87	0. 43	0. 64	1. 04	2. 56
J. V.	Reticulum-cell sarcoma (RCSA)	Alkylating agent	21	6. 7	2. 78	0. 30	0. 44	1. 02	2. 17
C. L.	Acute leukemia (AL)	None	20	5. 2	1. 91	0. 32	0. 87	1. 14	0. 94
J. F.	Multiple myeloma (MM)	Urethan	14	5. 8	2. 99	0. 42	0. 95	0. 28	1. 17
A. H.	"	Adr. st., alkylating agent	16	—	—	—	—	—	—
H. K.	"	X ray, urethan	27	—	—	—	—	—	—
P. P.	"	Stilbamidine analogue‖	20	—	—	—	—	—	—

B. Prolonged survival

R. A.	Chronic lymphatic leukemia (CLL)	Adrenal steroid	104+	6. 3	3. 01	0. 44	1. 01	1. 04	0. 80
H. Ha.	"	None	330+	8. 0	2. 74	0. 35	0. 55	0. 94	3. 43
N. L.	"	Alkylating agent	322+	6. 7	4. 58	0. 78	0. 46	0. 70	0. 68
J. M.	"	Adrenal steroid	34	7. 7	3. 26	0. 57	1. 00	1. 60	1. 30
J. P.	"	Alkylating agent	144+	6. 9	2. 26	0. 40	0. 76	1. 08	2. 01
W. P.	"	None	300 (died)	6. 4	3. 14	0. 33	0. 71	0. 93	1. 30
P. R.	"	"	408+	6. 8	3. 48	0. 39	0. 52	1. 29	1. 13
C. C.	Hodgkin's disease (HD)	Alkylating agent	206+	5. 5	1. 28	0. 53	0. 80	1. 00	1. 90
L. L.	"	X ray	66	—	—	—	—	—	—
M. L.	"	Adrenal steroid	106+	8. 3	3. 28	0. 58	1. 68	1. 25	1. 53
M. S.	"	None	>30	—	—	—	—	—	—
G. B.	"	"	>30	—	—	—	—	—	—
W. Ca.	Lymphosarcoma (LSA)	Alkylating agent	60+	6. 9	4. 55	0. 29	0. 49	0. 79	0. 80
E. M.	"	X ray	36+	—	—	—	—	—	—
M. I.	Reticulum-cell sarcoma (RCSA)	Adr. st., alkylating agent	210	6. 5	3. 43	0. 45	0. 81	1. 20	0. 63
J. K.	"	Alkylating agent	>40	—	—	—	—	—	—
M. Rub.	"	X ray, alkylating agent	210+	6. 0	2. 44	0. 29	0. 48	1. 01	1. 78
H. Hen.	Multiple myeloma (MM)	Adr. st., X ray urethan	59 (died)	—	—	—	—	—	—
M. O.	"	Antimetabolite¶	348 (died)	12. 2	2. 01	0. 36	0. 78	0. 68	8. 38

*Days shown are those on which evidence of rejection was first noted. Since patients were not seen every day, rejection may have occurred earlier. For prolonged-survival patients M. I. and M. O., the skin graft used was fetal in origin. + indicates graft still surviving.

†Adrenal steroid includes prednisone, cortisone, dexamethasone, and hydrocortisone.

‡Alkylating agent includes nitrogen mustard, chlorambucil, depoxypropylpiperazine, and epoxypropidine.

§Thioguanosine.

‖4-4'-Diamidinodiphenylamine dihydrochloride.

¶6-Mercaptopurine and 6-diazo-5-norleucine (DON).

TABLE 2.—Immunological reactions in patients showing typical rejection and prolonged survival of homografts

Patient	Diagnosis	Blood type	Isohemagglutinin titers		Agglutinin titers: Typhoid		Agglutinin titers: Paratyphoid		Skin test reactions			Properdin titer*
			Anti-A	Anti-B	O	H	A	B	Tuberculin	Mumps	*Candida albicans*	
A. Typical rejection												
J. D.	CLL	O	1:8	1:4	0	0	0	0	0	+	0	0
H. Her.	"	A	—	1:4	0	0	0	0	+	++	+	5(Z)
N. H.	"	—	—	—	—	—	—	—	++	0	++	—
S. L.	"	A	—	1:8	0	0	0	0	0	0	0	0
B. M.	"	—	—	—	—	—	—	—	+	0	+	0
C. Ri.	"	B	1:16	—	—	—	—	—	0	0	0	8
C. S.	"	O	1:4	1:250	0	1:40	1:80	1:160	0	++	0	5
N. S.	"	O	1:16	1:4	0	0	0	1:80	+	++	+	12
J. T.	"	O	1:4	1:2	—	—	—	—	0	0	0	5(Z)
W. Co.	HD	O	1:64	1:16	0	0	0	0	0	++	0	Trace
F. G.	"	B	1:16	—	0	0	0	0	+	++	0	8(Z)
H. M.	"	—	—	—	—	—	—	—	0	0	0	—
C. Ra.	"	A	—	1:4	0	0	0	0	—	—	—	1
G. S.	"	—	—	—	—	—	—	—	—	—	—	0
G. W.	"	—	—	—	—	—	—	—	0	0	0	—
C. M.	CML	—	—	—	—	—	—	—	0	+	0	—
A. Y.	"	—	—	—	—	—	—	—	0	0	0	3(Z)
H. Hei.	LSA	—	—	—	0	0	0	0	0	0	+	—
J. V.	RCSA	—	—	—	—	—	—	—	—	—	0	5
C. L.	AL	O	0	0	—	—	—	—	0	0	0	1
J. F.	MM	B	1:32	—	—	—	—	—	0	0	0	1
A. H.	"	—	—	—	—	—	—	—	0	0	—	—
H. K.	"	—	—	—	—	—	—	—	—	—	—	—
P. P.	"	—	—	—	—	—	—	—	—	—	—	—
B. Prolonged survival												

B. Prolonged survival

R. A.	CLL	A	—	1:4	0	0	0	0	+	0	0	5(Z)
H. Ha.	"	A	—	0	0	0	0	0	0	0	0	8
N. L.	"	O	1:16	1:8	0	0	0	0	0	0	+	3(Z)
J. M.	"	—	—	—	—	—	—	—	0	+	+	—
J. P.	"	B	1:4	—	0	0	0	0	0	+	+	8
W. P.	"	O	0	0	0	0	0	0	0	+	0	5
P. R.	"	A	—	1:16	0	0	0	0	0	+	+	0
C. C.	HD	—	—	—	—	—	—	—	0	—	0	2
L. L.	"	—	—	—	0	0	0	0	—	—	—	0
M. L.	"	A	—	1:4	0	0	0	0	0	0	0	—
M. S.	"	—	—	—	—	—	—	—	—	—	—	—
G. B.	"	—	—	—	—	—	—	—	—	—	—	—
W. Ca.	LSA	O	1:128	1:32	—	—	—	—	0	0	0	0
E. M.	"	—	—	—	—	—	—	—	0	0	0	—
M. I.	RCSA	B	0	0	—	—	—	—	—	—	—	0
J. K.	"	—	—	—	0	0	0	0	+	0	+	—
M. Rub.	"	—	—	—	0	0	0	0	0	0	0	8(Z)
H. Hen.	MM	—	—	—	—	—	—	—	—	—	—	—
M. O.	"	O	1:16	1:8	0	0	0	0	—	—	—	—

*Eighty percent of normal controls showed properdin titers in excess of 2. Zero indicates no detectable properdin at the 1 unit/ml level. Z = zone, which refers to the presence of an inhibitor to properdin present in high concentration of serum.

β-globulins was found in both groups of patients. Deficiencies in the level of albumin were also seen in both groups.

The isohemagglutinin titers in these 2 groups of patients are outlined in table 2. In the Typical Rejection group only 1 patient (acute leukemia) with Type O blood had no isohemagglutinins. Three of the 10 patients tested in the Prolonged Survival group failed to reveal appropriate isohemagglutinins.

The response to vaccination with typhoid-paratyphoid A and B vaccine is presented in table 2. There was a general deficiency in antibody response after this vaccination. The highest positive titer in a dilution of 1:20 or greater is recorded. Two of the 9 patients in the Typical Rejection group produced antibodies; in only 1 patient was the titer higher than 1:80 and this response was to 1 antigen only. None of the 11 patients in the Prolonged Survival group responded.

The results of skin testing the homograft recipients with tuberculin, mumps, and *C. albicans* antigens are presented in table 2. In the Typical Rejection group, 10 of the 19 patients were positive to at least 1 skin test. Of the 13 patients in the Prolonged Survival group tested, 7 were positive to at least 1 skin test.

The results of the determination of the level of properdin are listed in table 2. Eight of the 16 patients in the Typical Rejection group had low properdin titers compared to 5 of 11 in the Prolonged Survival group.

The results of skin heterotransplantation in 9 patients who had previously had skin homografts are presented in table 3. Six of these patients had had prolonged survival of homologous skin grafts. Patient W. P. accepted a skin heterograft for 33 days; he died of empyema and bronchopleural fistula on the 33d day. Figures 1*a*, 1*b*, 2, and 3 show the heterologous skin graft, a biopsy of the heterologous skin graft, and the homologous skin graft. When the heterograft was performed, the patient was receiving no therapy except antibiotics. As his infectious complications worsened, large doses of adrenocortical steroids were added with immediate but transient improvement. The adrenocortical steroids may have played a role in the acceptance of this graft. However, these agents were started 7 days after heterografting and up to that time there had been no evidence of an inflammatory reaction. In contrast, figures

TABLE 3.—Results of skin heterografting in patients with lymphomas who had previously received skin homografts

Patient	Diagnosis	Number of days homograft survived	Number of days heterograft survived
R. A.	CLL	104+	18
H. Ha.	CLL	330+	14
W. P.	CLL	300	33 (died)
J. P.	CLL	144+	14
W. Co.	HD	19	68
C. C.	HD	206+	20
M. Rub.	RCSA	210+	16
C. L.	AL	20	10
J. F.	MM	14	13

4*a*, 4*b*, 4*c*, and 4*d* show the inflammatory response to heterografting in patient C. L.

Patient W. Co. with Hodgkin's disease rejected a homologous skin graft in typical fashion in 19 days. Ten months later he accepted a heterologous skin graft for 68 days. The patient was treated with radiation therapy and adrenocortical steroids at the time of both grafting procedures. There was progressive general deterioration in his condition during the 10 months between the 2 procedures. A homologous skin graft was not applied subsequent to the application of the heterograft, due to the development of thrombocytopenia.

Serum was taken from 6 of these patients after rejection of the heterograft and studied for the presence of antibodies with the use of the gel diffusion technique (*9*). The serum was used undiluted. An aqueous extract of homogenized pigskin was used as the antigen. After lyophilization, it was prepared in merthiolated saline in 6 concentrations (from 0.5 to 20 mg/ml) for instillation in the antigen reservoir. No antibodies were demonstrated.

DISCUSSION

Since some patients with lymphomatous disease accept homografts for long periods, they may be able to benefit from the absence of the immune barrier to transplantation. Furthermore, knowledge of the immunological deficit breaching the barrier to homologous transplantation in disease may throw light on the normal function of this mechanism. It would also be useful to have a test to indicate whether or not a patient will accept a homograft. Such a test would be useful in the evaluation of other diseases where the barrier to transplantation is diminished, *e.g.*, uremia and advanced carcinomatosis. If such a test were quantitative, it could be used to evaluate the effects of treatment to overcome this barrier. Pretesting with skin, especially from the intended donor, may be inadvisable since it could lead to accelerated rejection or second-set reaction against the subsequent graft (*10*).

None of the tests performed in this study correlated with acceptance or rejection of the transplant. All 4 patients with hypogammaglobulinemia promptly rejected their homografts. In these patients with lymphomas, there was a general deficiency in antibody production with no significant distinction between the 2 response groups.

The failure of skin testing to correlate with the results of grafting is significant since the skin tests employed and the reaction to homologous transplantation are both believed to be due to the same immune mechanism, delayed hypersensitivity. A note of caution is appropriate about the use of intermediate strength (0.0002 mg) tuberculin test antigen (PPD). There were 8 instances in which this test was negative while either the mumps or *C. albicans* skin test was positive. There was only 1 case where this test was positive and the other 2 negative. As part of an-

other procedure, patient W. E., who was negative to intermediate strength PPD, was tested with a second-strength dose. A strongly positive reaction developed. An intermediate-strength dose is said to detect 73.4 percent reactors and first and second-strength doses, 76.8 percent (*11*). To obtain maximum correlation between tuberculin testing and skin graft, it appears desirable to use the second-test strength (0.005 mg) if the intermediate is negative. Since no quantitative test of delayed hypersensitivity is available, one cannot say how sensitive these skin tests are *a posteriori*, they are not as sensitive as skin grafting.

In the studies of Southam and Pillemer, carcinoma patients who accepted tumor homografts generally had a properdin deficiency (*2*). This was not true in the present study of patients with lymphomas.

It has been shown that alkylating agents and adrenal steroids are able to prolong the survival of skin homografts (*13*, *14*). Patients in both the Typical Rejection and Prolonged Survival groups received therapy with these agents. In general, there was no significant difference in the treatment received by patients in either group, nor was there any consistent difference in the general clinical condition of either group.

Each diagnosis was represented in both response groups except for acute leukemia and chronic myelogenous leukemia in which there were no prolonged survivals. It might be expected that in these myeloproliferative diseases there would be less interference with the immune response than in the lymphoproliferative diseases and Hodgkin's disease. However, the small number of cases in the myeloproliferative group does not permit any such generalization.

No antibodies were found in the serum of 6 patients who had received heterologous skin transplants. Possibly, by the use of immune techniques more sensitive than gel diffusion they would have been found, since antibodies to heterologous transplantation have been demonstrated with other systems (*15*).

The criteria used in this study for estimating rejection and survival of the graft were repeated gross inspection for graft necrosis or progressive contracture and scar formation; admittedly these criteria are not optimal. While these signs are distinct enough for reliable observation, there may also be instances of "creeping rejection" of a graft with replacement of donor epithelium by that of the recipient with no sharp endpoint detectable. Distinctive sex chromatin in the epithelial cells would be a satisfactory marker for such situations and in the future we shall use this method whenever possible.

In homologous transplantation of immunologically competent tissue, such as bone marrow or lymph nodes, delayed death of the recipient frequently occurs, which is known as homologous disease or secondary syndrome. This has been variously interpreted as graft-versus-host reaction and host rejection of the graft. In these patients with lymphomas who accept homografts, a unique opportunity may be present to contribute to the solution of this dilemma, since presumably a host-versus-graft reaction could not occur.

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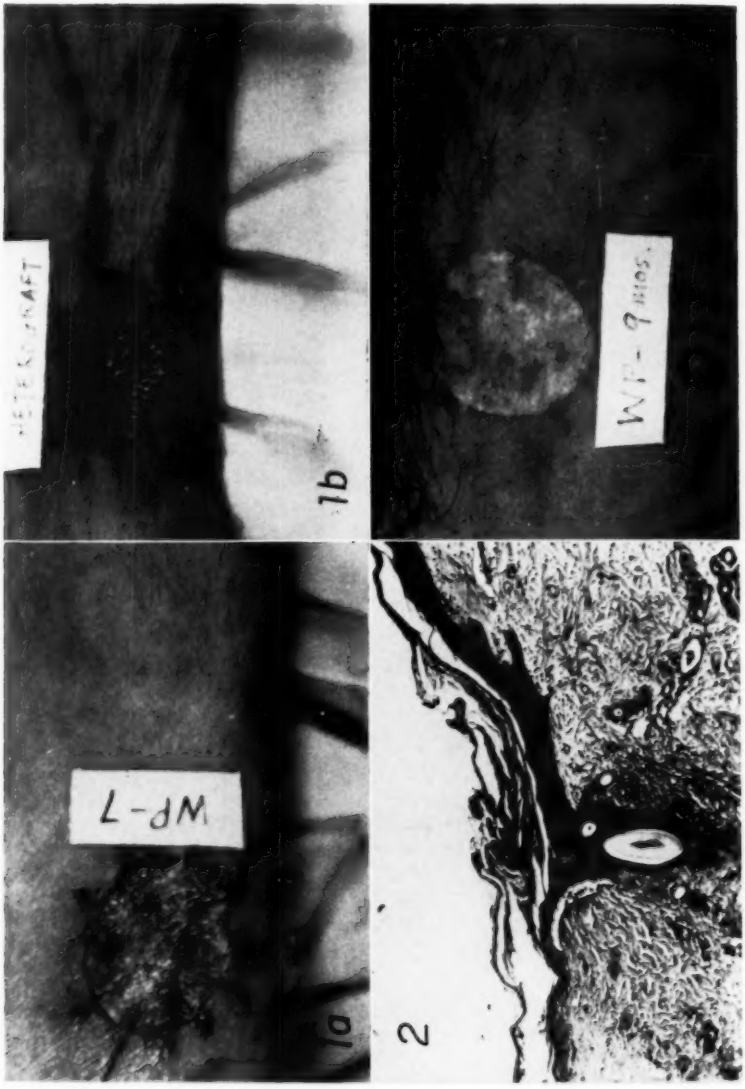
PLATES



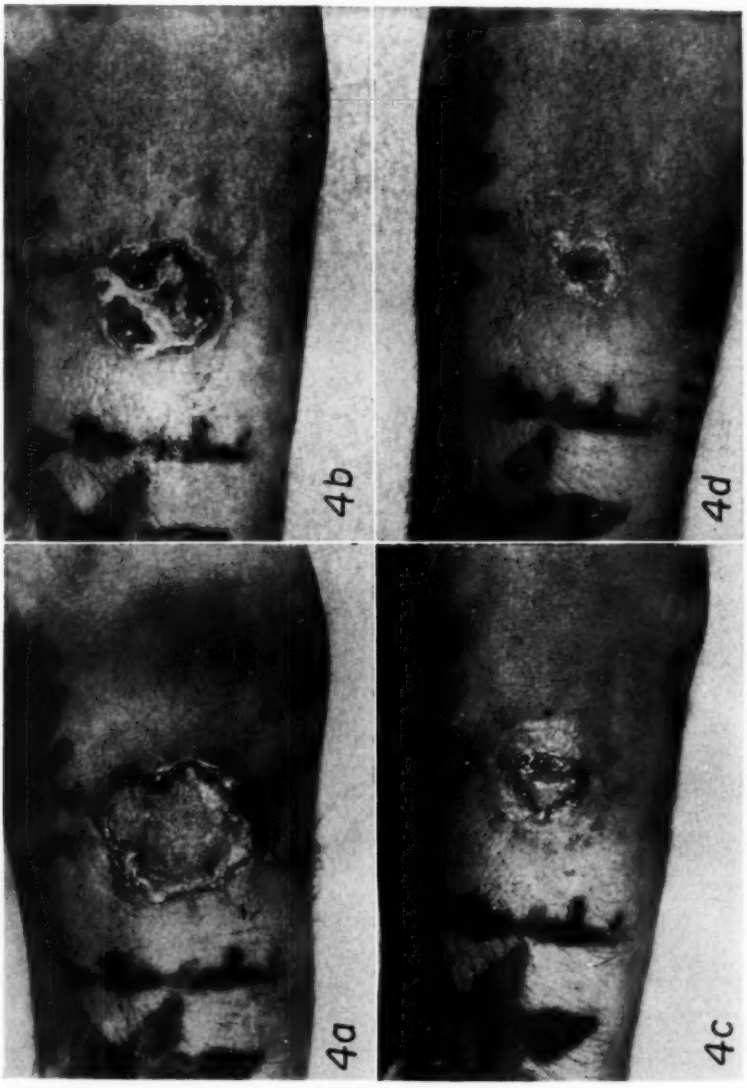
MILLER *et al.*

PLATE 81

FIGURE 1.—Pigskin graft in patient W. P. with chronic lymphatic leukemia (*a*) at 7 days, and (*b*) at 22 days.

FIGURE 2.—Biopsy of pigskin graft in patient W. P. at 8 days. Photomicrograph at junction of donor's and recipient's skin. × 40

FIGURE 3.—Skin homograft in patient W. P. at 9 months.



MILLER *et al.*

PLATE 82

FIGURE 4.—Pattern of rejection of pigskin graft in patient C. L. with acute leukemia (*a*) at 7 days, (*b*) 14 days, (*c*) 21 days, and (*d*) 28 days.

Cytotoxicity of Heterologous Immune Chicken Serum to Normal and Neoplastic Mouse Tissues in Culture [1, 2]

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SUMMARY

New Hampshire Red cockerels were immunized against the Sarcoma 180 mouse tumor and against tissues of normal mice of its BALB/c host. Plasmas and serums from immunized and nonimmunized chickens were used in tissue cultures of the tumor and of embryonic mouse tissues. Antibodies against the tumors showed cytotoxic effects on tumor cells even after absorption with normal mouse antigens, but did not affect embryonic cells. Similar effects were not shown by antibodies against normal mouse tissues.—J. Nat. Cancer Inst. 26: 585-599, 1961.

DURING RECENT years there has been a number of attempts to demonstrate the presence of specific antigenic components in tumorous tissues (*2–5*). The results of these studies have generally not resolved whether the presumed tumor-specific antigens may be specific merely to the tissue of tumor origin. There has accumulated, however, a considerable body of evidence (*6, 7*) which points to the antigenic distinctiveness of various organs and tissues, and it would not be surprising to find tumors showing antigenicity that differed from normal cells of their hosts. Should it be possible to produce cytotoxic antibodies against distinctive tumor antigens, there could possibly be developed a powerful tool for bringing about the selective destruction of tumor cells *in vivo*.

In most previous studies, the rabbit has been used as antibody producer and, in those instances in which cytotoxic effects of tumor antiserums have been demonstrated, complement has been a necessary part of the reaction mixture (*8–15*). Nonspecific cytotoxins for tumor cells have been demonstrated in normal serums by several investigators (*16–19: cf.*, however, *20–23* for contrary evidence) and thus, cytotoxic effects of antitumor

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[3] The authors gratefully acknowledge the technical assistance of Mr. Joseph Balsano.

580132—61——9

serums, even after appropriate absorption procedures, may not be indicative of the presence of antibodies specific to the tumor.

Accordingly, the present study has been designed to test whether the fowl can produce cytotoxic antibodies against antigens distinctive of tumor cells and whether cytotoxic effects in tissue culture might be a more sensitive and, possibly, a more reliable method for detecting specific antibodies than the customary immunological procedures. For this study, the mouse tumor Sarcoma 180 (S180) was chosen. Although it is to be expected that this tumor would be somewhat different antigenically from tissues of its host strain by virtue of its long history of transplantation, it was chosen for the initial tests because of ready availability, rapid growth, and amenability to simple tissue-culture techniques.

Antiserums produced by the chicken have been tested for effects on the growth of tumor cells *in vitro* either with or without prior treatment by appropriate absorbents. Cytotoxic effects have been obtained after absorption of the antiserums with antigens prepared from tissues of normal mice. The results strongly suggest that antibodies are produced in the chicken against antigenic components of the tumor which are not shared by other tissues of the host strain.

MATERIALS AND METHODS

Tumor.—The transplantable S180 mouse tumor which originated in 1914, was procured from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, where it is routinely carried in mice of the BALB/c strain. The tumor stock obtained from the Jackson Laboratory was maintained by mouse-to-mouse transplant. The transplants were performed aseptically to prevent tumor contamination with bacterial antigens. Tumors were harvested for preparation of the injection antigen approximately 3 weeks after implantation.

Preparation of tissue for immunization.—Antigens were prepared from tumors and from tissues of nontumorous mice of the inbred host strain. In the early phases of this project, freshly excised tumors were finely minced, suspended in 5° C 1 percent NaCl (20 g of tumor in 100 ml of the salt solution), homogenized for 1 minute at high speed in a chilled Waring blendor, and centrifuged for 30 minutes at 1900 $\times$ *g*. The precipitated material was then ground for 2 minutes in a Potter-Elvehjem homogenizer, resuspended in the original supernatant, and treated for 1 minute in the Waring blendor. The resulting brei was filtered through several thicknesses of cheesecloth and used immediately as antigen.

Recently, we used a more convenient procedure. Freshly excised tumors were frozen at −20° C and stored until needed. Prior to use, the tissue was thawed, finely minced with scissors, suspended in 5° C 0.9 percent NaCl, and homogenized for 2 minutes in a chilled Waring blendor. The tissue pulp was then frozen rapidly over dry ice and acetone and dried *in vacuo* for 18 to 25 hours. The dried tissue was powdered and

stored at −20° C. For use it was made up as a 4 percent suspension in 0.9 percent NaCl, treated in a Waring blendor for 1 minute, and centrifuged at 1900 × *g* for 30 minutes. The supernatant of this preparation was used immediately as an antigen. Both antigen preparations were equally effective in eliciting antibody responses in the chicken. Furthermore, antiserums prepared against each type of antigenic preparation were identical in both titer and cytotoxic effects on tumor cells in tissue culture. In view of the greater convenience with the use of powdered antigens, the second method of tissue preparation is now routinely used.

We employed the same procedures to prepare antigenic material for absorption and control purposes from normal tissues of mice of the inbred host strain. All tissues of the mouse were homogenized together ["whole-mouse homogenate" (*24*)] and used as sources of normal tissue antigens. Thus proper controls were provided for the possibility that one might be dealing with organ-specific rather than tumor-specific antigens. This is particularly important, for the tissue and site of origin of the S180 tumor are unknown. Normal mice were killed each time tumor preparations were made, and their tissues were processed concurrently with the tumors.

Production of antiserums.—Antiserums against tumorous and normal tissues were produced in New Hampshire Red cockerels, 5 to 6 months of age and weighing 2500 to 3000 g. Nonlyophilized antigens were injected intravenously in the wing vein with 1 ml of the homogenate per kg of body weight. For lyophilized antigens, 3 ml of the 4 percent antigen solution was injected per bird.

As previously shown (*24*), the use of other injection routes, with or without adjuvants, does not improve the antibody titer. In most cases, however, 1 or 2 secondary injections were given at 3- to 5-week intervals to maintain the antibody titer and to provide an adequate supply of antiserums.

Blood was drawn from fasting, immunized birds by cardiac puncture. The antiserums were separated by centrifugation and stored at −20° C for subsequent analysis by serological and tissue-culture methods.

Analytical methods.—The serological titration procedure in this study was fully described in an earlier publication (*24*).

In vitro *tests.*—Tumors were freshly excised aseptically, transferred to a sterile dish containing Earle's balanced salt solution (*25*) with streptomycin (0.2 mg/ml) and penicillin (0.12 mg/ml), and trimmed of adhering host tissues. Slices of tumor 1 mm in thickness were prepared and necrotic areas dissected out. Firm tumor tissue was then cut into cubes approximately 1 mm on a side. Fine fragments were placed separately on each glass coverslip and to each was added a drop of the appropriate plasma and a drop of embryonic extract prepared by the syringe method (*26*) from 9-day chick embryos. The tumor thus grew at the glass-clot interface and was observed microscopically at intervals for 72 hours.

In other experiments, tumor tissues were explanted to plasma-clot cultures made from the blood of nonimmunized birds. These cultures regularly showed vigorous growth. After 24 to 48 hours, "immune plasma" was added to the culture and the response of the outgrowing cells was recorded by means of time-lapse cinemicrography.

For further tests, cell suspensions of the tumor were prepared by trypsinization and cultured on Eagle's medium (*27*) fortified with 10 percent horse serum. Cultures grown on the glass surface of standard milk-dilution bottles were then trypsinized and transferred to 35 mm Carrel flasks. Vigorously growing cells of the first transplant generation were treated with absorbed or nonabsorbed antiserums and the responses of the cells were followed microscopically.

OBSERVATIONS

Effects of Plasmas From Birds Immunized With Tumor Antigens

The initial series of experiments was designed to compare the growth of tumor fragments explanted on media containing "tumor-immune plasma" with the growth of similar explants on media containing plasma of nonimmunized birds. In all, 456 tissue cultures were prepared using immune plasmas from a total of 20 birds. Primary bleedings were chiefly used as a source of plasma, and antibody titers ranged up to 1:16. Whenever possible, controls for these cultures were grown on media prepared from plasma taken from the same bird before the injection of antigen or, at least, from nonimmunized birds of the same strain.

A total of 301 control cultures was made over a period of 3 years. Of these, 96 percent showed vigorous fibroblastic outgrowth after 24 hours, and only 1.6 percent failed to show growth after 72 hours. In these cultures, the characteristic outgrowth comprised large numbers of smooth, spindle-shaped tumor cells which commenced migration within a few hours and rapidly spread centrifugally (fig. 1). From these observations it is clear that the plasma of the normal fowl contains no natural antibodies or factors, *e.g.*, complement, which inhibit the normal behavior of tumor cells in culture.

In contrast, of the 456 cultures prepared from plasmas of immunized birds, 81 percent showed no outgrowth after 24 hours, but the remaining 19 percent exhibited some centrifugal migration, usually of rounded or oval cells (fig. 2). After 72 hours, however, 37 percent of the cultures showed at least some outgrowth of cells, many typically spindle-shaped. The results are strongly suggestive of inhibition of tumor growth by immune plasma.

It is suggested that complement may not participate in this inhibitory effect. To the extent that a complete or partial complement might be present in chicken plasma, it would not be expected to retain its activity under the conditions of prolonged storage to which all plasmas were subjected (*6*, *7*). Complement-fixation tests with sensitized sheep red

blood cells have shown low levels of complement in serum from freshly drawn New Hampshire Red chicken blood, but revealed no detectable amounts in preparations stored for as little as 24 hours under refrigeration (8° C) or for several months in the frozen state (−20° C).

As one would expect (*13*, *28*, *29*), no correlation was found between the inhibitory effects of the immune plasmas and their precipitin titers, for there is no *a priori* reason why the cytotoxic antibody has to be part of a precipitating system. Indeed, of the 28 plasma preparations used in this study, 7 showed no interfacial titers, and the others showed titers of 1:2 to 1:16. Interestingly, of 7 plasmas showing no titer, 4 completely inhibited outgrowth of the cultures. In contrast, outgrowth occurred in cultures made from some plasmas which showed appreciable titers.

The question next arises whether immune plasmas can exercise a cytotoxic effect on vigorously growing cells in plasma-clot cultures made with normal plasma. Accordingly, coverslip preparations of tumor cells were grown in the presence of normal plasma for approximately 48 hours. A drop of immune plasma was then added to the preparation, and the resulting effects were observed directly through the microscope and by means of time-lapse cinemicrography. Peripheral cells of the outgrowth lying at the glass-clot interface were chiefly affected; the central and deeper-lying cells were unaffected. Characteristically the cells withdrew their protoplasmic processes within a matter of 10 minutes, and showed a rounded periphery with densely aggregated refractile granules around the nucleus and with a hyaline cortical zone. The latter had an intense "bubbling" activity, rapidly protruding and withdrawing clear vesicles. Within 20 to 25 minutes, this bubbling activity ceased and the cells became quiescent (figs. 3 through 8). No further activity was noted during several days of subsequent observation.

It is clear from these observations that plasmas of birds receiving intravenous injections of S180 preparations have adverse effects on tumor cells in tissue culture. One might now inquire whether the outgrowth of normal cells of the host mouse strain would also be inhibited under similar conditions. To test this possibility, a variety of tissues from 15-day embryonic mice of the BALB/c strain, including heart, skin, nervous system, sense organs, and mesenchyme was explanted in cultures made with normal cockerel plasmas and with immune plasmas previously shown to be inhibitory to tumor cells. The cultures regularly showed outgrowth in both plasmas, which thus indicated that embryonic cells, at least, are unaffected by immune plasmas. Similar tests have not yet been carried out on tissue cultures of normal adult cells.

Effects of Plasmas From Birds Immunized With Antigens of Normal Mouse Tissues

In a further series of 326 experiments, tumor fragments were confronted in cultures, similar to those previously described, with plasmas prepared from cockerels immunized with antigens from homogenates of

normal mouse tissues. Luxuriant outgrowth regularly occurred, but in 36 percent the onset of centrifugal migration of the cells was delayed as much as 24 hours; in only 16 percent was there no outgrowth at all. It is of particular interest to note that, although antibody titers to normal mouse tissues were as high or higher (1:16 to 1:32) than in those in which plasmas of birds immunized with tumor antigens were used, there was, nevertheless, no comparable degree of inhibition of the cultures. This suggests that there are antigenic differences between tumorous and normal tissues of the mouse.

Effects of Plasmas From Birds Immunized With Antigens From Heterologous Mouse Tumors

The question may be raised whether plasmas from birds immunized against other tumors would also inhibit the growth of S180 cells in culture. To test this possibility, plasmas from birds immunized with S91 melanoma antigens (*cf. 24*) were used in tissue-culture media for a total of 53 explants. The explants regularly showed outgrowth, though there was sometimes a short delay, comparable with that observed when plasmas from birds immunized with antigens of normal mouse tissues were used.

Effects of Absorbed Antiserums on Growth of S180 Cultures

The foregoing experiments clearly indicate that adverse effects of plasmas from birds immunized with antigens of the S180 tumor are due to the presence of antibodies specific for tumor antigens. This being true, it would be expected that antiserums produced against the tumor and absorbed with normal mouse-tissue antigens would show inhibitory or cytotoxic effects on tumor cells *in vitro*. Accordingly, pooled antiserums against the tumor were absorbed exhaustively as follows: 4 ml of pooled antiserum against the S180 tumor was absorbed with 2 ml of normal mouse antigen, prepared as previously described, and 2 ml of 14 percent NaCl was added to give a final salt concentration of 8 percent in the reaction mixture. The latter was incubated for 1 hour at 37° C and then refrigerated at 4° C overnight. If, after centrifugation, the addition of 0.25 ml of the antigen to 1 ml of the supernatant elicited no further precipitation, it was assumed that antibodies against normal mouse-tissue components of the tumor had been absorbed. If a precipitation did occur, small amounts of antigen were again added to the serums and treated as before. Special care was taken to avoid the addition of excess antigens to reduce the possibility of nonspecific absorption of antibodies and excessive dilution of the antiserums.

Freshly excised tumors, trimmed of host tissues, were minced and trypsinized, 0.5 percent trypsin in alkaline Hanks' solution (*30*). Resulting cell suspensions were washed and cultured in milk-dilution bottles with Eagle's medium. After 8 days the cells were harvested, resuspended in Eagle's medium, and placed in Carrel flasks. Each flask received approximately 750 cells in 0.8 ml of medium. After 72 hours, 0.25 ml

of absorbed antiserum was added. Control cultures received a like amount of normal chicken serum and observations were begun immediately. Within 5 minutes there were clear indications of a cytotoxic reaction in cells of the cultures that received the absorbed antiserum; as in the hanging-drop cultures described, cellular processes were withdrawn and the cell bodies rounded up. In control cultures no adverse effects were observed.

DISCUSSION

These results clearly indicate that antiserums against the S180 tumor show cytotoxic effects on cells growing in culture, even after complete absorption with antigenic components from all normal mouse tissues. These observations are in close agreement with those of Björklund *et al.* (*28*, *31*) using horse antiserums and those of Filatov (*32*) using goat antiserums.

Many workers (*16–19*) have demonstrated the presence of nonspecific cytotoxins for tumor cells in normal serums. Indeed, Bolande and McClain (*33*) and others (*34*) have shown the presence of heat-labile cytotoxins for S180 cells in normal human serum. On the other hand, several investigators (*20–23*) have been unable to demonstrate cytotoxic activity in normal serums. McAllister *et al.* (*20*) demonstrated that cytotoxic activity to HeLa cells was not present in normal serums from cows, humans, monkeys, rabbits, rats, goats, or guinea pigs. Green and Lorincz (*35*) have described the presence of a natural antibody, in the blood of chick embryos, capable of destroying intravenously injected Krebs ascites tumor cells. On the other hand, Bueker (*36*) and Levi-Montalcini *et al.* (*37*) have reported excellent growth of the S180 tumor after one or more passages on the chorio-allantoic membrane of the chick embryo. Our studies are in agreement with the latter workers in that no cytotoxic activity was detectable to S180 cells grown in normal chicken serums. Indeed, 96 percent of a total of 301 control cultures grown on such normal serums showed vigorous fibroblastic outgrowth after 24 hours of incubation.

Several investigators have found that complement is required for the cytotoxic activity of antiserums to tumor cells in culture (*8–15*). However, Horn (*38*) prepared a rabbit antiserum against the soluble phase of Ehrlich ascites cells, which possessed a high cytotoxic activity against these cells *in vivo* and *in vitro*. He found that a major portion of this activity was complement-independent, a minor portion complement-dependent. Similarly, Mountain (*23*) prepared an antiserum against ruptured HeLa cells, which had an agglutinating activity that was complement-independent. Miller and Hsu (*19*), using homogenized HeLa cells, obtained rabbit immune serums which proved to be complement-independent for the cytotoxic effect. Ebert (*39*) and Clayton (*40*), studying the effects of antiorgan serums on chick and newt embryos,

respectively, found specific cytotoxic effects after heat inactivation of complement. In the present study it also appears that the cytotoxic effect of chicken-immune serums on mouse tumor cells may be complement-independent. Further studies are being directed to this issue.

As with any tumor having a long history of transplantation, it is not unexpected that antigenic differences between tumor and host tissues should be detected by sufficiently sensitive methods. It is particularly encouraging, however, to find that the cytotoxic or inhibitory effect of antitumor antibodies offers what is probably a more sensitive test of antigenic distinctness between tumor and host tissue than that which is provided by the usual serological procedures. One is encouraged by this result, therefore, to apply these techniques to tumors of more recent origin which can be cultivated *in vitro* by routine procedures. Already there are hopeful indications [current experiments; also (*41*)] of antigenic distinctness between tumor and host in the more recent tumors, BW10232 (mammary adenocarcinoma) and B16 (melanoma), but suitable methods for the routine culturing of these tumors *in vitro* for the purpose of assaying cytotoxicity have not been devised.

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PLATES

PLATE 83

FIGURE 1.—Characteristic outgrowth of tumor explant after 24 hours on plasma from nonimmunized bird.

FIGURE 2.—Slight centrifugal migration of cells from periphery of tumor explant after 72 hours *in vitro* on plasma from cockerel previously immunized with tumor antigens.

1

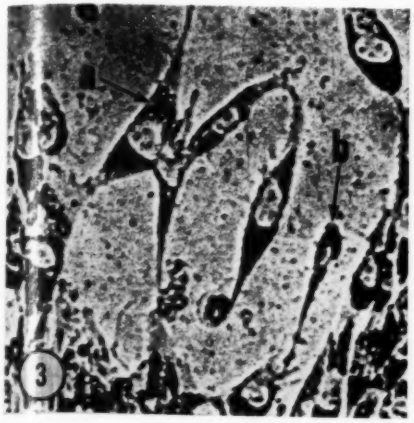
2

PLATE 84

Cytotoxic effects of immune plasma on S180 cells *in vitro*.
Enlargements from time-lapse 16 mm film sequence.

FIGURE 3.—Appearance of peripheral cells after 48 hours on medium prepared from plasma of nonimmunized bird.

FIGURES 4 TO 8.—Progressive changes recorded at intervals of 5, 15, 25, 35, and 45 minutes after addition of immune plasma to culture. Cells designated a and b showed marked changes within 5 minutes. Other cells responded more slowly but, eventually, all cells in this field withdrew their protoplasmic processes and, after a period of intensive cortical "bubbling," became quiescent.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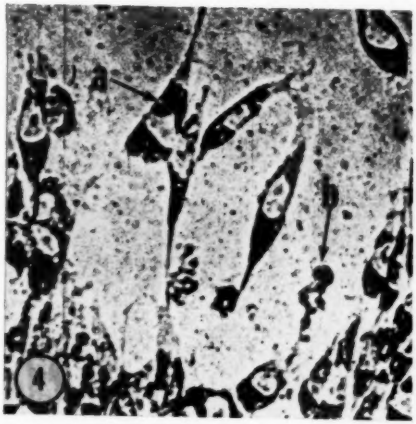
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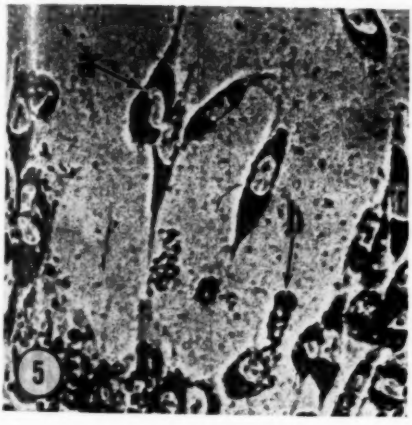
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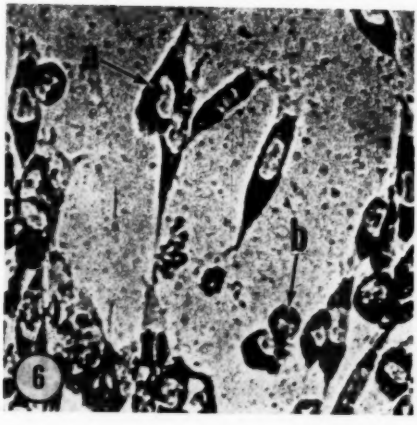
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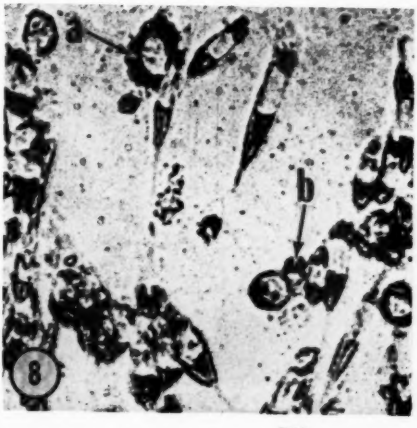
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Effect of Cortisone on Growth, DNA, RNA, and Protein of Spontaneous and Transplanted Mammary Tumors in Mice [1]

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SUMMARY

The influence of cortisone upon the growth and metabolism of spontaneous and transplanted mammary tumors in C3H mice has been studied. The inhibition of tumors by cortisone was more striking in transplanted than in spontaneous tumors, as has been reported, but this could not be attributed to differences in growth rates. Some transplanted tumors in mice 10 months of age did not respond to cortisone treatment, in contrast to transplants in young mice. An important factor in determining the responsiveness of these tumors to cortisone is the age of the host. The level of nucleic acids and protein in tumors varied to the same extent in all groups. Marked variation characterized the *in vivo* incorporation of glycine-2-C^{14} and leucine-1-C^{14} into tumor protein and of adenine-8-C^{14} into nucleic acids, and no consistent biochemical differences could be demonstrated between spontaneous and transplanted tumors, with or without cortisone treatment, by the methods used in this study.—J. Nat. Cancer Inst. 26: 601–610, 1961.

THIS INVESTIGATION was undertaken to search for possible biochemical differences between spontaneous and transplanted tumors. The original plan was for serial observations on spontaneous tumors followed through several transplant generations, but it was interrupted by loss of the early transplant generations during a laboratory move. An alternate plan was to exploit the known difference in biological response of spontaneous and transplanted tumors to chemotherapeutic agents such as cortisone (*1*). The growth of first- and second-generation transplants was reported to be inhibited by a number of compounds, including cortisone, but the spontaneous tumors from which these transplants originated were relatively insensitive. Several strains of mice and over a score of compounds were tested, and the difference appeared to be a general phenomenon (*2*, *3*). Scholler *et al.* (*1*) suggested that

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580132—61——11

observed differences between growth rates of spontaneous and transplanted tumors might explain the difference in response.

Genetic and cytological variation could be minimized by the use of mammary tumors indigenous to an inbred strain of mice. The choice of biochemical parameters to be measured presented difficulties. Ribonucleic acid (RNA)/deoxyribonucleic acid (DNA) and protein/DNA ratios, and the incorporation of labeled substrates into these compounds were chosen in view of their importance in growth and cell duplication and the possibility of extending initial observations. The objectives of the experiments were not attained, but the observations may be of value to others in their design of future experiments.

MATERIALS AND METHODS

Animals and tumors.—Female C3H/Jax mice, obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, were used exclusively. The mice were housed in plastic cages, with water and mouse pellets available at all times. The spontaneous tumors were mammary adenocarcinomas, confirmed by histological examination. Transplanted tumors were derived from the spontaneous tumors by subcutaneous trocar implantation into the axillary region of a 1 mm^3 fragment. A different spontaneous tumor was used as a donor for each experiment. In experiments 10 through 14 a common donor was used for the transplants into young and old mice.

Tumor measurements were made with calipers in 2 dimensions, and tumor weights calculated from Schrek's formula (*4*), the third diameter being the mean of the 2 diameters measured. Tumors were approximately 5 mm in diameter at the beginning of an experiment, which was of 10 days' duration. Cortisone (Cortone, Merck & Co., Inc.) was injected subcutaneously every 2d day for 5 doses, at a level of 50 mg/kg of body weight. The number of tumors which grew so that the sum of 2 diameters increased by more than 6 mm in 10 days is tabulated. The growth index of tumors which grew by at least this amount is defined as the ratio of tumor weight at the end of an experiment to tumor weight at the beginning, as described by Martinez *et al.* (*5*).

Chemicals.—Radioactive substrates were obtained from the Atomic Energy of Canada, Ltd., Ottawa, Ontario, and were produced by the Radiochemical Centre, Amersham, Buckinghamshire, England. The specific activities were as follows: adenine-8-C^{14}, 6.2 mc/mmole; glycine-2-C^{14}, 1.1 mc/mmole; leucine-1-C^{14}, 3.3 mc/mmole. Labeled compounds were injected intraperitoneally in 0.1 ml of 0.9 percent NaCl, at a dose of 2×10^6 counts/minute/mouse at 9:00 a.m., and the mice killed by cervical dislocation 6 hours later. Radioactivity was measured in a Nuclear Corporation Model D46A gas-flow G-M counter, with planchettes 7.2 cm^2

in area. The total material on any planchette never exceeded 3 mg, and it was unnecessary to correct for self-absorption.

Chemical methods.—Tumor tissue was removed immediately after the mice were killed. The tissue was trimmed free of necrotic and hemorrhagic areas, a fragment fixed for histological examination, and the remainder ground in 10 volumes of cold, distilled water. An aliquot was removed for estimation of protein by a micro-Kjeldahl method (*6*) or by the Folin-Ciocalteu phenol reagent (*7*). Trichloroacetic acid (TCA) was added to a final concentration of 2 percent, the samples centrifuged, and the supernatant, together with 1 wash, collected. An aliquot of this fraction was assayed for total nucleotides and radioactivity, and the total purines calculated (*8*). The remainder was passed through a Dowex-50 cation exchange resin, washed through with 4 ml of 1 N HCl as described by LePage (*9*), and the concentration and radioactivity of the nucleotides determined as noted later. The tissue precipitate after removal of the acid-soluble fractions (ASF) was extracted once with 80 percent ethanol and once with ethanol-ether (1 : 1) at 70° C for 10 minutes to remove lipides, and the extracts were discarded.[4] The residue was extracted overnight at 0 to 4° C in 10 volumes of 1 N perchloric acid (PCA) to remove RNA, as described by Ogur and Rosen (*10*). The DNA was then extracted with 5 percent TCA at 90° C for 30 minutes. Nucleotides were determined by ultraviolet (UV) absorbency at 265 mμ in a Beckman DU spectrophotometer, and when necessary we removed TCA by heating samples in a boiling water bath for 2 hours. Aliquots of the RNA and DNA fraction were reserved for colorimetric determination of ribose and deoxyribose, as described by Barnum *et al.* (*11*), to permit correction for cross-contamination. Frequently some RNA was found in the DNA fraction, but as the specific activities of both were of the same order, the resulting error was accepted. The RNA fraction was heated at 90° C for 30 minutes, cooled to 55° C, and 0.1 volume of 20 percent $AgNO_3$ added. The samples were stored overnight at 0 to 4° C. The precipitate of the silver salts of purines was collected by centrifugation, washed with cold 0.1 N H_2SO_4, and redissolved in hot 0.1 N HCl. Total purines were measured by UV absorbency at 252 mμ, according to Loring *et al.* (*12*). Each fraction was heated in a beaker to reduce the volume to approximately 1 ml, plated directly onto a stainless steel planchette, dried, and counted. Duplicate determinations were made on each tumor, and agreement within 15 percent was obtained.

For the determination of protein-specific activity, the tumor tissue was extracted with hot PCA, 80 percent alcohol, hot alcohol-ether, and the extracts were discarded. The residue was washed with acetone, dried *in vacuo* overnight, and suspended in 25 percent ethanol in a tissue grinder. Portions were plated onto tared aluminum planchettes, dried, weighed, and counted.

[4] No appreciable activity was found in these extracts with adenine-8-C^{14} or leucine-1-C^{14}, but considerable activity was noted from glycine-2-C^{14}.

RESULTS

The data presented in tables 1 and 2 are in general agreement with Scholler *et al.* (*1*) in that the effect of cortisone is more marked on the transplanted tumors ($P = <0.001$ by the chi-square test). However,

TABLE 1.—Effect of cortisone on spontaneous mammary tumors

Experiment No.	Controls			Cortisone-treated*		
	Number† which grew/ total	Mortality‡	Body-weight change (g)	Number† which grew/ total	Mortality	Body-weight change (g)
1	3/8	1	+0.2	4/10	2	−2.5
2	6/11	—	+1.2	4/11	1	−0.2
3	5/8	—	+1.4	4/10	1	−1.8
4	6/8	—	+1.0	5/15	3	−2.0
5	4/9	—	+1.0	0/4	2	−1.7
6	10/14	—	+1.6	2/4	2	−2.2
Total 1–6	34/38			19/54		
	Growth index of 34 tumors which grew: 5.5 ± 0.8(SEM)§			Growth index of 19 tumors which grew: 5.0 ± 0.7(SEM)		

*Dosage: 50 mg/kg body weight of Cortone injected subcutaneously every 2d day for 5 doses.
†Number of tumors which increased at least 3 × 3 mm in 10 days.
‡Not included in totals.
§Standard error of the mean.

TABLE 2.—Effect of cortisone on transplanted mammary tumors* in young mice (6–10 weeks)

Experiment No.	Controls			Cortisone-treated†		
	Number‡ which grew/ total	Mortality	Body-weight change (g)	Number‡ which grew/ total	Mortality	Body-weight change (g)
1	7/8	—	+1.0	1/6	1	−1.0
2	5/7	—	+1.8	1/7	—	−0.7
3	4/5	1	+2.4	0/6	—	−3.5
4	7/7	—	+2.0	2/12	1	−2.0
5	9/12	—	+1.5	0/7	—	−0.6
6	6/8	—	+1.8	0/7	—	−1.4
7	5/7	—	—	1/5	—	—
8	3/3	—	—	1/4	—	—
9	4/4	—	—	2/6	—	—
10	7/8	—	+1.3	1/8	1	−1.7
11	2/6	—	—	0/9	—	—
12	12/15	—	—	1/33	—	—
13	6/8	—	+1.4	1/8	1	−1.7
14	4/5	—	+1.7	0/10	—	−2.5
Total 1–14	81/103			11/128		
	Growth index of tumors which grew: 6.6 ± 0.9(SEM)			Growth index of tumors which grew: 3.2 ± 0.9(SEM)		

*All tumors were first-generation transplants, from a different spontaneous tumor in each experiment.
†Dosage: 50 mg/kg body weight of Cortone injected subcutaneously every other day for 5 doses.
‡Number of tumors which increased at least 3 × 3 mm in 10 days.

comparison of the cortisone-treated with the control spontaneous tumors (table 1) indicates an effect of cortisone at the 0.05 level of significance, when the data from all 6 experiments are pooled.

The extent of the response was not in relation to body-weight loss. Mortality was higher among mice bearing spontaneous tumors, and deaths were not included in calculations. The growth index indicates that spontaneous tumors had growth rates similar to those of untreated first-generation transplants. A fourth-generation transplant was observed to be growing more slowly than spontaneous tumors. Eight of 26 controls and only 1 of 28 cortisone-treated mice achieved the criterion of growth noted in the tables. Though the difference does not attain a level of significance, it suggests that a cortisone response may be occurring in slowly growing transplanted tumors.

Since spontaneous tumors arise in old mice and transplants are usually carried in young mice, the influence of the age of the host on the response of its tumor to treatment was investigated. Tables 2 and 3 show that tumors differ in their susceptibility, but the effect of cortisone on the transplanted tumors was usually less in older mice.

An examination of the data on cortisone-treated transplants in older mice in table 3 suggests two separate populations—experiments #10, 13, 14, and 15 demonstrate a lack of response to cortisone, and experiments #11, 12, and 16 show a definite effect of cortisone treatment on the transplants in older mice. Comparison with the same experiments in table 2 indicates a consistent effect of cortisone on the transplants in young mice.

We selected tumors for chemical studies after a 10-day period of treatment, which was begun 4 to 5 weeks after grafting. From each group a representative tumor was selected that was grossly free of hemorrhagic and necrotic areas and which had responded in a manner typical of that group.

TABLE 3.—Effect of cortisone on transplanted mammary tumors in old mice (9–12 months)

Experiment No.†	Controls			Cortisone-treated*		
	Number‡ which grew/total	Mortality	Body-weight change (g)	Number‡ which grew/total	Mortality	Body-weight change (g)
10	5/6	1	+0.5	3/5	2	−3.1
11	—			0/5	—	−2.2
12	—			1/32	4	—
13	—			6/6	—	−1.7
14	5/7	1	+1.4	6/8	1	−0.4
15	9/11	—	+1.1	11/12	—	−2.2
16	10/10	—	+0.9	5/18	3	−2.0
Total 10–16	29/34			32/86		
	Growth index of tumors which grew: 6.5 ± 0.9(SEM)			Growth index of tumors which grew: 5.5 ± 0.8(SEM)		

*Dosage: 50 mg/kg body weight of Cortone injected subcutaneously every other day for 5 doses.
†Experiments numbered to coincide with table 2; each number represents a different spontaneous tumor donor.
‡Number of tumors which increased at least 3 × 3 mm in 10 days.

The RNA/DNA and protein/DNA ratios of tumors are recorded in table 4. No significant differences were found between spontaneous and transplanted tumors, or between cortisone-treated and untreated tumors. The DNA content per unit of wet weight was the same for spontaneous and transplanted tumors.

TABLE 4.—RNA/DNA and protein/DNA of tumors

Group	RNA/DNA*	Protein/DNA†
Spontaneous tumor	1. 09 ± 0. 15 (10)‡	1. 09 ± 0. 22 (10)
Spontaneous tumor + cortisone§	1. 14 ± 0. 20 (7)	1. 00 ± 0. 19 (7)
Transplanted tumor	1. 19 ± 0. 17 (10)	0. 94 ± 0. 22 (10)
Transplanted tumor + cortisone	1. 20 ± 0. 20 (10)	0. 84 ± 0. 26 (10)

*RNA/DNA ratios were obtained by pentose determinations.

†Protein was estimated by the Folin-Ciocalteu reagent and expressed as mg of tyrosine.

‡Mean ± standard error of the mean. Number of mice shown in parentheses.

§Dosage: 50 mg/kg body weight of cortisone acetate (Merck & Co., Inc.,) injected subcutaneously every other day for 5 doses.

The uptake of labeled glycine and leucine into tumor protein is shown in table 5. Transplants incorporated more glycine than spontaneous tumors, but this was not evident in the uptake of leucine. Cortisone decreased the incorporation of leucine into tumors which were inhibited by cortisone, *i.e.*, transplanted tumors in young mice.

TABLE 5.—*In vivo* incorporation of glycine-2-C^{14} and leucine-1-C^{14} into tumor protein

Group	Specific activity, counts/min./mg protein	
	Glycine-2-C^{14}*	Leucine-1-C^{14}
Spontaneous tumor	183 ± 14 (24)†	699 ± 110 (10)
Spontaneous tumor + cortisone‡	—	826 ± 130 (7)
Transplanted tumor:		
In young mice	260 ± 8 (24)†	795 ± 130 (11)†
In young mice + cortisone	—	280 ± 49 (11)†
In old mice	—	528 ± 94 (10)
In old mice + cortisone	—	356 ± 64 (14)

*Experiments with glycine-2-C^{14} were based on an earlier series of mice.

†$P<0.01$, between groups indicated by dagger. Mean ± standard error of the mean. Number of mice shown in parentheses.

‡Dosage: 50 mg/kg/body weight of cortisone acetate injected subcutaneously every other day for 5 doses; 2×10^6 counts/min./mouse of glycine-2-C^{14} or leucine-1-C^{14} injected intraperitoneally 6 hours prior to killing.

The results of labeling tumor nucleic acids by adenine-8-C^{14} are given in table 6. Transplants tended to incorporate more adenine into RNA and DNA[5] than spontaneous tumors, and cortisone treatment decreased labeled DNA in transplanted tumors at the 0.05 level of significance. It is doubtful that such an approach is meaningful. In the group of spontaneous tumors treated by cortisone, individual tumors which did

[5] The total acid-soluble purines and the nucleotides were analyzed to relate the activity of nucleic acids to that of the presumed "precursor pool" in the same tumor. Calculating the "relative specific activity," *i.e.*, the ratio of specific activity of the nucleic acid to that of either nucleotides or total acid-soluble purines did not alter the conclusion.

TABLE 6.—*In vivo* incorporation of adenine-8-C^{14} into nucleic acids of tumors

Group	Specific activity, counts/min./mg purine	
	RNA*	DNA†
Spontaneous tumor	427 ± 100 (15)‡	104 ± 15 (15)
Spontaneous tumor + cortisone§	254 ± 61 (11)	83 ± 20 (11)
Transplanted tumor	858 ± 170 (14)	243 ± 74 (14)
Transplanted tumor + cortisone	509 ± 153 (12)	73 ± 19 (12)

*Spontaneous vs. transplants $P<0.05$.

†DNA purine was calculated by UV absorbency at 265 mμ. Spontaneous vs. transplants $P = 0.05$; transplants vs. transplants + cortisone $P < 0.05$.

‡Means ± standard error of the mean. Number of mice shown in parentheses.

§Dosage: 50 mg/kg/body weight of cortisone acetate injected subcutaneously every other day for 5 doses; 2×10^6 counts/min./mouse adenine-8-C^{14} was injected intraperitoneally 6 hours prior to killing.

and did not respond were selected, and the incorporation of adenine-8-C^{14} compared. It is obvious from an examination of table 7 that there is no demonstrable difference between the 2 groups at the biochemical level.

The variation encountered in all groups, however, makes interpretation difficult. In the course of this work it was noted that a group of tumors in the sixth-transplant generation exhibited less variation in labeled RNA and DNA. To establish whether this was due to repeated transplantation or to the common origin of the grafts, a group of first-generation transplants all derived from the same spontaneous tumor were examined. Grafts also were made from a long-established transplantable mammary adenocarcinoma, C3H/Ba, indigenous to the C3H strain. The range of

TABLE 7.—*In vivo* incorporation of adenine-8-C^{14} into nucleic acids of cortisone-treated spontaneous tumors

Group	Specific activity, counts/min./mg purine	
	RNA	DNA
Tumor growth inhibited	259	47
	272	55
	24	4
	81	9
	683	210
Not inhibited	170	57
	56	13
	262	95
	431	298
	279	72
	283	59

adenine incorporation for the various groups is compared in table 8. In contrast to a 22-fold variation among spontaneous or transplanted tumors chosen at random, only a threefold range was found where transplants were derived from a common source. This emphasizes the biochemical variation even among tumors of similar morphology and genetic background.

TABLE 8.—Variation in the *in vivo* incorporation of adenine-8-C^{14}* by various tumors

Tumor	Specific activity, counts/min./mg purine	
	RNA	DNA†
10 Spontaneous tumors	70–1, 360‡	39–238
14 Transplanted tumors	86–1, 911	45–1, 046
6 First-generation transplants from a single primary tumor	892–2, 142	323–762
7 Transplants from C3H/Ba tumor	551–1, 472	235–619

*Dosage: 2×10^6 counts/min./mouse of adenine-8-C^{14} injected intraperitoneally 6 hours prior to killing.
†DNA purine was calculated by UV absorbency at 265 mμ.
‡The range of values is indicated.

DISCUSSION

Changes in tumors during transplantation have been discussed by Dunn (*13*) and Foulds (*14*). Transplantation of a spontaneous tumor results in considerable selection of cell types (*15*). However, Hauschka (*16*) has stressed the heterogeneity of tumor-cell populations; the capacity for temporary or permanent alterations is present even in long-transplanted tumors.

It is possible that, during transplantation, selection reduces the cell types present in the transplant as compared with those in the spontaneous tumor. Why the transplants should be more responsive to a variety of therapeutic agents, however, is not evident. The present data suggest that the difference in response to cortisone is quantitative, and the cortisone does have some slight effect on the spontaneous tumor.

Stock (*17*) has discussed the usefulness of transplanted tumors in chemotherapeutic screening trials, because of greater uniformity in behavior and reproducibility of results in such trials. The present data, while agreeing with this, indicate that considerable variability is still present among transplanted tumors of similar histological type within an inbred strain of mice, and underline the known difference in response between spontaneous and transplanted tumors.

The importance of the vascular supply in solid tumors has been stressed by Woglom [cited by Dyer (*18*)] and by Algire and Chalkley (*15*), who have proposed that the growth of a tumor is dependent upon the persistent ability to evoke formation of a vascular system. The influence of various agents on the formation of the tumor vascular system, provided by the host, could conceivably differ between young, growing mice and mature mice.

The greater effectiveness of cortisone in suppressing transplants in young mice as compared with transplants in some old mice suggests a host influence modifying the response of the tumor. This might explain some of the differences observed between spontaneous tumors and transplants (*1*, *17*), but the data presented in table 3 show variability from experiment to experiment in the response of transplanted tumors in older mice to cortisone, and directs attention once more to variability in the tumor donors.

This problem may be relevant to the resistance to steroid therapy observed in human cancer (*19*, *20*). It is possible that numerous factors in the tissues of the host as well as in the tumor can influence the maintenance of dose levels sufficient to suppress tumor growth.

Greenstein (*21*, *22*) has emphasized the biochemical similarity of diverse tumors and the tendency to converge to a common endpoint. Changes observed in tumors during transplantation suggest a loss of specialized functions as the tumors tend toward a common metabolism. In mouse hepatomas a stepwise decrease in the concentration of the enzyme cystine desulfurase and an increase in the activity of alkaline phosphatase occurred with progressive transplantation. Greenlees and LePage (*23*) observed that a mouse mammary tumor acted as a "nitrogen trap" (*24*) only after five transplant generations.

The attempt herein to relate behavior and chemistry of tumors having presumably an identical genetic and cytological composition has not been successful. The gross composition of tumors was variable to the same extent in all groups for any parameter measured. The incorporation of labeled amino acids into protein and of adenine into nucleic acids tended to be higher in transplanted than in spontaneous tumors, which possibly reflects the somewhat enhanced growth of transplants (*15*). Cortisone decreased the labeling to a greater extent in transplants, the growth of which was inhibited by treatment, but the differences were not sharply defined and of questionable significance. Variation was great, particularly in adenine incorporation, and appears to be a true biological variation. It is of interest that similar variation has been noted in human breast tumors, even under *in vitro* conditions (*25*).

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Effects in Newborn Mice of Tumor-Producing Agents Carried in Tissue Culture [1, 2]

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SUMMARY

Cells and extracts of a variety of spontaneous and induced mouse and human tumors were inoculated into mouse-embryo tissue culture. Inoculated and noninoculated tissue-culture fluids were tested for tumor-inducing ability by injection into newborn mice. Ten of 41 cultures inoculated with mouse tumor cells or extracts and 3 of 21 cultures inoculated with materials from cancer patients produced parotid tumors in injected mice. These included cultures inoculated with AKR spontaneous leukemia; X ray plus estrogen-induced C57 leukemia; X ray plus estrogen plus urethan-induced C57 leukemia; methylcholanthrene plus orotic acid-induced DBA leukemia; X ray plus urethan-induced C57 leukemia, induced (C3Hf × AKR)F_1, (C3H × AKR)F_1 and C3Hf parotid-gland tumors, and transplanted DBA parotid-gland tumor; bone marrow of 2 patients with granulocytic leukemia. Seventy-five mice receiving injections of noninoculated tissue-culture fluid and 80 mice receiving no treatment and kept in the same area did not develop parotid tumors, though a high percentage of those tested were found to have positive polyoma virus hemagglutination-inhibition (HI) titers. Sera of 5 mice with parotid-gland tumors induced by fluids from cultures inoculated with human material (1 from each of the "positive" patients) or with irradiation and chemically induced mouse leukemia were tested for polyoma virus HI and showed high titers. Polyoma virus was identified in one of the tissue-culture fluids which had been inoculated with leukemic human bone marrow and which had induced parotid tumors when injected into newborn mice. The significance of these findings with respect to attempts to isolate human tumor viruses is discussed.—J. Nat. Cancer Inst. 26: 611-619, 1961.

MUCH EVIDENCE for tumor viruses in mice has been accumulated, particularly in recent years, by Gross, Friend, Stewart, Moloney, Graffi, and others, (*1–7*), and reviews covering this literature have appeared

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(*8–12*). The use of newborn test animals for leukemia studies by Gross and the application of tissue-culture methods by Stewart and her associates represent important contributions. The inoculation of newborn animals with tissue-culture fluids of mouse parotid tumors caused the production of multiple tumors after a much shorter latent period and in species other than the mouse (*3–5*).

Our studies were initiated with the following objectives: 1) to confirm the experiments by Stewart, Eddy, and their associates; 2) to extend these studies to chemically induced and transplanted neoplasms; and 3) to extend the tissue-culture method to human neoplasms. These studies were begun in August, 1957. Since that time the serologic method of detection of polyoma virus hemagglutination-inhibiting (HI) antibody was reported, and made possible epidemiological studies of polyoma virus that have had an important influence on the investigation of tumor viruses by cell-free extracts or tissue-culture methods (*13–19*).

This report is concerned with: 1) the effects in newborn mice of tissue-culture fluids inoculated with various mouse and human neoplastic tissues; 2) the distribution of polyoma virus HI antibodies among the mice used for bioassay; 3) demonstration of polyoma virus in the tissue-culture fluids; and 4) the difficulties encountered in the bioassay of human materials and its interpretation.

MATERIALS AND METHODS

Materials for inoculation into tissue culture were obtained from both mice and patients with neoplastic diseases as follows: 1) cells or extracts of tissues—thymus, lymph node, spleen, liver, tumor, etc.—of mice with spontaneous or induced leukemia, parotid-gland tumors, mammary tumors, and various transplanted tumors; and 2) bone marrow, peripheral blood, feces, and postmortem tissues—brain, liver, spleen, and lymph node—of patients with leukemia, Hodgkin's disease and lymphoma.

For tissue-culture monolayers, monkey kidney cells (Microbiological Associates, Inc., Bethesda, Md.), and chorio-allantoic membranes from embryonated chicken eggs were used at first. In one case, cells from whole AKR embryos were used. Later, C3H/Bi mouse embryos from our laboratory were consistently used. The embryos were minced and trypsinized after removal of the eyes. Resulting cell suspensions were cultivated in prescription bottles in a medium consisting of 0.5 percent lactalbumin hydrolysate and 2 percent calf serum in Hanks' solution. For the propagation of polyoma virus the cells were maintained in a medium consisting of 0.25 percent lactalbumin hydrolysate and 1 percent horse serum in Earle's solution.

Solid materials were minced in petri dishes and 2 loopfuls, 3 mm in diameter, were inoculated into each of 2 tissue-culture bottles. Extracts were prepared by the following procedures: Materials were ground in a mortar, made up into 20 percent suspension with saline solution, centri-

fuged at 3000 rpm for 10 minutes at 0° C; the supernatant was recentrifuged at 7000 rpm for 5 minutes at 0° C and passed through a Selas #02 porcelain filter. Aspirated bone marrows were diluted tenfold with saline and inoculated into tissue-culture bottles in the same way as minced solid materials. Unless cytopathogenic effect was seen, the cultures were kept in an incubator of 37° C for 14 days. During this period, the medium was changed when it became acid. Harvested fluids of all inoculated and noninoculated tissue cultures were injected into newborn mice, mostly (C3Hf × AKR)F_1 hybrids. The procedures employed were similar to those used by Stewart *et al.* (*3*, *4*).

Polyoma virus tissue-culture fluid for hemagglutination studies was kindly supplied by Dr. B. E. Eddy, of the National Institutes of Health, and recultured in our laboratory.

RESULTS

Bioassay of Tissue-Culture Fluids Inoculated With Mouse and Human Neoplasms

Six hundred and thirty mice survived longer than 1 month after injection at birth with tissue-culture fluids inoculated with either mouse or human tumor material. Fifty-seven of these mice, 9 percent, developed parotid tumors with or without other tumors such as lung tumors, leukemia, kidney tumors, and mammary tumors at 2 to 12 months of age (table 1).

Ten of 41 of the tissue-culture fluids inoculated with mouse neoplastic materials induced parotid tumors with or without other tumors in mice that had received injections. These 10 had been inoculated with AKR spontaneous leukemia; X ray plus estrogen-induced C57 leukemia; X ray plus estrogen plus urethan-induced C57 leukemia; methylcholanthrene plus orotic acid-induced DBA leukemia; X ray plus urethan-induced C57 leukemia; transplanted DBA parotid tumor induced with polyoma virus; (C3Hf × AKR)F_1, (C3H × AKR)F_1, and C3Hf parotid tumors induced with various tissue-culture fluids of these mouse neoplastic tissues and the human neoplastic tissues described in the next paragraph.

Three of 21 tissue-culture fluids inoculated with materials from cancer patients induced parotid tumors with or without other tumors such as lung, kidney, and mammary tumors in injected mice; all 3 were inoculated with bone marrows aspirated from patients with acute granulocytic leukemia, of which 2 were from the same patient aspirated at a 15-day interval.

Seventy-five mice receiving injections of the noninoculated tissue-culture fluid and 80 mice receiving no treatment and kept in the same area did not develop parotid or any other tumors except leukemia. Leukemia of apparently spontaneous nature occurred in approximately equal incidence in all groups of experimental and control animals.

Distribution of Polyoma Virus Antibodies Among Bioassay Mice

In the later stages of this study the HI method for the detection of antibodies against polyoma virus became available, together with evidence for environmental dissemination of polyoma virus from infected mice (*13–17, 20*). It became important, therefore, to survey the polyoma virus HI status of the serums of both treated and untreated mice in the experimental area.

TABLE 1.—Production of parotid tumors with tissue-culture fluids inoculated with neoplastic materials

Neoplastic material inoculated into tissue culture	Number of materials	Number of materials that produced parotid tumors	Number of mice surviving	Number of mice developing	
				Parotid tumors	Other tumors
From mouse:					
Leukemia, spontaneous: cells or extract	9	2			
Leukemia, induced: cells or extract	12	4			
Parotid-gland tumor: cells	6	3			
Mammary-gland tumor: cells or extract	9	0			
Transplanted tumor: cells	5	1			
Total	41	10	393	45	41 Leukemia 9 Mammary 2 Subcutaneous 1 Lung 1 Muscle
From human:					
Bone marrows aspirated from leukemia, Hodgkin's disease, and lymphoma	15	3*			
Others—peripheral blood, tissue, and feces	6	0			
Total	21	3	237	12	20 Leukemia 3 Mammary 2 Lung 1 Kidney
Noninoculated control tissue culture	17	0	75	0	8 Leukemia
Noninoculated mice			80	0	6 Leukemia

*Of 12 parotid tumors induced with these 3 materials, 1 was not confirmed microscopically.

The HI test was therefore done with serums of representative mice of several groups: mice bearing induced parotid tumors, nontumorous cage mates of parotid tumor-bearing mice, control mice receiving injections of the noninoculated tissue-culture fluid, and untreated mice. Details of the methods and results are reported elsewhere (*21*).

All 5 tested mice bearing parotid tumors had high polyoma virus HI antibody titers. These mice had received injections of fluid from tissue cultures inoculated with (*a*) leukemic bone marrow from each of the 2 "parotid tumor-induction-positive" cancer patients, and (*b*) irradiation

and chemically induced mouse leukemic tissue. Their 12 inoculated but nontumorous cage mates showed positive, though somewhat lower, titers. However, 22 mice inoculated with fluids from control tissue cultures and 20 nontreated mice also showed a high percentage of positive but still lower titers, 73 percent and 85 percent respectively. It appeared from these data that polyoma virus was highly disseminated among these mice.

Demonstration of Polyoma Virus in Tissue-Culture Fluids Inoculated With Leukemic Human Bone Marrow

To resolve the question of whether the positive results with human materials were caused by (*a*) an agent unmasking latent polyoma-virus infection in the test mice, (*b*) polyoma virus itself, or (*c*) an antigenically related human virus, the following experiments were undertaken:

As a first step, one of the frozen, −65° C, tissue-culture fluids that had been inoculated with bone marrow of a leukemic patient and which had induced 5 parotid tumors in 7 inoculated mice, was recultured. An agent, which was cytopathogenic for mouse-embryo tissue-culture cells and agglutinated guinea-pig red cells, was demonstrated in the recultured tissue-culture fluid (table 2).

TABLE 2.—Tissue-culture repassages of frozen parotid-tumor-inducing tissue-culture fluid originally inoculated with human leukemic bone marrow

Material inoculated into tissue culture	Parotid-tumor incidence	HA* before reculti-vation	1st Tissue-culture repassage				2d Tissue-culture repassage	
			10 days		12 days		5 days	
			CPE†	HA	CPE	HA	CPE	HA
Tissue-culture fluid‡	5/7	—	—	—	++	1:32	+++	1:160
Control			—		—	—	—	—

*HA = Hemagglutination with guinea-pig red cells.

†CPE = Cytopathogenic effect.

‡This is the 2d passage—before freezing—tissue-culture fluid; the fluid of 1st passage induced parotid tumors in none of 7 inoculated mice, that of the 2d passage in 5 of 7, and that of the 3d passage in 4 of 15.

For the identification of this agent the HI test was performed with the tissue-culture fluid of the isolated agent and known polyoma virus as antigens, and with sera of the patient from whom the "tumor-induction-positive" bone marrow was aspirated, and known polyoma virus HI-positive mouse sera as antibodies (table 3). Two sera of the patient were taken at about 3-months' interval. One inhibited both the isolated agent and known polyoma virus to a dilution of 1:20. The other sample of patient's serum was negative at 1:10. In contrast, one of the polyoma-immune mouse sera inhibited both antigens to a dilution of 1:400. The other mouse serum inhibited the isolated agent to 1:1600 and known polyoma virus to 1:3200, a one-tube difference.

TABLE 3.—HI test with hemagglutinating agent recovered from parotid-tumor induction-positive tissue-culture fluid

	Serum	Antigen	HI titer
Patient*	Serum taken 5–27–59	Recultured tissue-culture fluid	1:20
		Polyoma virus tissue-culture fluid	1:20
	Serum taken 8–12–59	Recultured tissue-culture fluid	<1:10
		Polyoma virus tissue-culture fluid	<1:10
Polyoma virus HI positive mice	Serum of BALB/c ♂	Recultured tissue-culture fluid	1:400
		Polyoma virus tissue-culture fluid	1:400
	Serum of CE ♂	Recultured tissue-culture fluid	1:1600
		Polyoma virus tissue-culture fluid	1:3200

*The tissue-culture fluid inoculated with this patient's bone marrow induced parotid tumors in mice that were inoculated, and a hemagglutinating agent was recovered from this tissue-culture fluid.

DISCUSSION

The production of tumors in mice by tissue-culture fluid inoculated with parotid-tumor cells and spontaneous leukemic cells of mice, reported by Stewart and Eddy (*3–5*) and others, was confirmed. In 4 of 12 instances, mice receiving injections of tissue-culture fluid inoculated with leukemic tissue induced in the C57 and DBA strains by X-ray, estrogen, urethan, and other chemical agents, also developed parotid tumors. However, 3 such mice with parotid tumors were tested and showed high HI titers against polyoma virus. Evidence for induction of lymphoid tumors by cell-free filtrates of radiation-induced lymphoid tumors has been reported by others (*22*, *23*).

Regarding the positive results obtained from tissue cultures inoculated with human leukemic marrow, one might wishfully be led to assume that these parotid tumors were induced by an agent isolated from the patients because: (*a*) Two of the "positive cultures" were inoculated with bone marrow obtained from the same patient at different times; (*b*) no parotid tumors arose among 75 mice receiving injections of 17 different noninoculated control tissue cultures and among 80 noninjected control mice. However, sera of 2 mice with parotid tumors induced by fluids from cultures inoculated with human material (1 from each of the 2 "positive" patients) were tested for polyoma HI and both showed high titers. This must be accepted as strong evidence that both tumors were induced by polyoma virus of mouse origin. Even this could not be concluded with certainty from this observation alone in view of the fact that immune sera of some viruses will inhibit the hemagglutinins of related viruses of another species. Thus both ectromelia and vaccinia hemagglutinins are inhibited by either vaccinia or ectromelia-immune serum.

If these tumors were the result of polyoma virus of mouse origin, the facts listed under (*a*) and (*b*) are suggestive that the tissue-culture fluid inoculated with human leukemic cells may have been instrumental in

unmasking or otherwise permitting the expression of polyoma virus of mouse origin.

These questions were largely resolved, however, by the reculture studies of a frozen tissue-culture fluid from the patient responsible for 2 of the 3 "positive" samples, and by the HI studies with this patient's serum. The finding, in the recultured fluid, of an agent that would hemagglutinate guinea-pig red cells and was inhibited by polyoma-immune mouse serum indicates that the culture contained either polyoma virus of mouse origin or a closely related and cross-reacting virus of human origin. The latter possibility appears unlikely in light of the absence of significant inhibition of either the isolated agent or known polyoma virus by the patient's serum.

If the isolated agent is indeed polyoma virus of mouse origin, the question arises of how it gained access to this culture, *i.e.*, via the mouse-embryo cells used for tissue culture, or via laboratory contamination of initially "clean" mouse-embryo tissue cultures. As measured by tumor induction, the apparent absence of polyoma virus from the great majority of the 21 cultures inoculated with human materials and from all the 17 control tissue cultures indicates the improbability or infrequency of prenatal infection of mice with polyoma virus. The epidemiology of polyoma-virus infection in laboratory mice also suggests postnatal rather than prenatal transmission (*17*, *21*, *24*). With respect to laboratory contamination of tissue cultures, Rowe has indicated that in work with polyoma virus it early became apparent that the frequency of cross contamination in tissue cultures and mice is much in excess of that previously experienced (*24*).

The need for strict isolation of any search for human tumor viruses from comparable studies of animal tumor viruses is apparent. It would also appear desirable to use a continuous culture of animal or preferably human cells for tissue culture to minimize the likelihood of introduction of animal viruses.

Burton *et al.* (*25*) have recently reported a high incidence of parotid tumors among C3Hf mice inoculated at birth with a "tumor factor" extracted from both mouse and human neoplastic tissue, with no tumors appearing among the controls. Unfortunately the polyoma virus HI status of the parotid-tumor mice and of the nontumorous controls was not reported.

The occurrence of polyoma antibodies among a high percentage of nontreated control mice in close proximity to mice inoculated with mouse parotid-tumor agent is also of great importance in the interpretation of the present data. It would appear from these data and those of Rowe *et al.* (*17*) that parotid-tumor agent from infected mice is disseminated into the environment. It would further appear that mice infected from the environment are most likely to develop antibodies without parotid-tumor formation, since none of 155 tissue-culture control and noninoculated control mice developed parotid tumors, but a high percentage of those tested showed positive HI titers. After the present newborn inoculations were performed, the rapid HI test for polyoma antibodies became available,

and the development of resistance to tumor induction by polyoma virus as a result of passive transfer of maternal antibodies was reported (*26, 27*). Environmental dissemination of the parotid-tumor agent is therefore probably more likely to result in "false negative" than in "false positive" tumors, particularly if the young of infected mothers are used for test purposes. Unfortunately the antibody status of the mothers of the newborn used in the present study is not known. The importance of such knowledge in any future work is obvious.

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Distribution of Hemagglutination-Inhibiting Antibodies Against Polyoma Virus in Laboratory Mice [1, 2]

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SUMMARY

Polyoma-virus antibodies were determined by the hemagglutination-inhibition (HI) method in two colonies of mice. In colony A, where mice were kept for breeding and experiments, 19 percent of 217 untreated mice of all ages had polyoma-virus HI-positive sera. No significant correlation existed between sex or strain of mice and HI titers. Untreated mice younger than 6 months were uniformly negative. In colony B, where newborn mice were extensively inoculated with various tissue-culture fluids, all mice bearing induced parotid tumors and their cage mates had positive sera and 54 percent of 41 untreated mice of all ages had positive sera. In a group of 153 mice grafted with any one of 25 transplantable tumors carried in colony A, 32 percent of mice of all ages had positive sera. From 5 of the 25 transplantable tumors, polyoma virus was actually isolated by tissue-culture propagation and identified serologically. Among mice bearing transplants of these 5 tumors, the percentage of positive HI tests ranged from 66 to 100 percent.—J. Nat. Cancer Inst. 26: 621-628, 1961.

SINCE THE development of serological methods for the detection of polyoma-virus antibodies in animals by Dr. Bernice Eddy, of the National Institutes of Health, and coworkers, reports have appeared on the epidemiological behavior of this virus (*1–5*). It becomes increasingly evident that polyoma-virus infection is an important consideration in laboratory mouse colonies, particularly where work with filtrates or tissue-culture fluids of mouse or human neoplasms is carried on (*6–9*).

Work with filtrates of mouse and human neoplasms has been in progress in this laboratory for 4 years, and with tissue-culture fluids inoculated with mouse and human neoplasms for 2½ years. Because parotid tumors developed in some of the mice used for bioassay (*6*), it became essential to survey the prevalence of polyoma virus in our mice by means of the newly developed hemagglutination-inhibition (HI) technique.

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This report is concerned with the frequency of positive polyoma-virus HI sera among mice (*a*) in areas where few experiments with neoplastic extracts have been carried on, (*b*) in areas where tissue-culture fluids inoculated with various neoplasms have been extensively injected into newborn mice, and (*c*) in mice bearing various transplantable tumors.

MATERIALS AND METHODS

Mice.—The laboratory mice of two colonies were tested. Colony A is a fairly large one in which mice were kept for breeding and various experiments, including work with filtrates of leukemic tissues of mice and human beings, and transplantation of various tumors. Colony B is housed in a rather small room located in a building separate from colony A. Newborn mice of colony B received extensive injections of various tissue-culture fluids which resulted in the development of parotid tumors in a moderate number (*6*). The mice of colony B or their ancestors were originally transferred from colony A.

Polyoma virus.—Polyoma virus tissue-culture fluid was kindly supplied by Dr. Eddy and recultured in our laboratory (*6*).

The HI test.—The test was done as described by Rowe *et al.* (*2*). Sera with HI titer equal to or higher than 1:200 were taken as positive (*2*). Mice were bled by inner canthus puncture or heart puncture.

Recovery of polyoma virus from transplanted tumors.—Tumor tissue was minced and 1 loopful, 3 mm diameter, was inoculated into 2 bottles of mouse embryo tissue culture in the same way that polyoma virus was inoculated (*6*). The presence of viral activity was indicated by serially transmissible cytopathogenic effect. Polyoma virus was identified serologically by hemagglutination of guinea-pig erythrocytes and by specific inhibition of such hemagglutination by serum of mice immune against known polyoma virus which was received from Dr. Eddy.

RESULTS

In colony A, 217 untreated normal mice of 22 strains or sublines were tested. The age, sex, and strain distribution of positive polyoma-virus HI titers is shown in table 1. Nineteen percent of tested mice of all ages showed positive HI titers. Of 33 mice over 12 months of age, 48 percent were positive, while 29 percent of 85 mice 6 to 12 months of age were positive. The 99 mice younger than 6 months of age were uniformly negative. No significant correlation appears to exist between strain or sex of mice and the frequency of HI positive sera. No correlation was observed between age and HI titers of positive mice, as shown in table 2.

In colony B, newborn mice received extensive injections of tissue-culture fluids inoculated with mouse and human neoplastic tissues, and 57 of 630 inoculated mice developed parotid tumors (*6*). The mice were (C3Hf ×

TABLE 1.—Frequency of positive HI titer in mice of colony A by strain, age, and sex

Strain	Age in months			All ages	Sex	
	<6	6–12	>12		♀	♂
BALB/cf	0/8	1/6		1/14	1/7	0/7
BALB/c	0/4		0/6	0/10	0/5	0/5
CBA	0/6	2/4		2/10	1/2	1/8
DBAf	0/9		3/3	3/12	2/4	1/8
DBA	0/11			0/11	0/7	0/4
DBAff		0/6	0/3	0/9	0/5	0/4
A	0/4	1/3		1/7	0/3	1/4
CE	0/4	2/4		2/8	0/2	2/6
NH	0/4	2/3		2/7	2/4	0/3
C3Hf	0/5	2/2	3/3	5/10	5/8	0/2
C3H	0/3	0/9		0/12	0/5	0/7
C3Hf/Gs	0/4	1/4	3/6	4/14	1/4	3/10
AKR	0/13	5/7	1/1	6/21	4/13	2/8
C58	0/6	2/7		2/13	1/5	1/8
C57BL	0/4	1/4	2/4	3/12	2/7	1/5
YBR	0/3	1/4		1/7	1/6	0/1
STOLI	0/3		3/4	3/7	1/3	2/4
FB	0/4	0/7		0/11	0/6	0/5
RIII	0/4	0/1	1/3	1/8	0/4	1/4
T6		0/5		0/5	0/5	
AK/n		2/4		2/4	2/2	0/2
(C3Hf × AKR)F_1		3/5		3/5	2/3	1/2
Total	0/99	25/85	16/33	41/217	25/110	16/107
Percent	0	29	48	19	23	15

TABLE 2.—Age and HI titer of positive mice in colony A

Age in months	Number of mice	HI titer				
		1 : 200	1 : 400	1 : 800	1 : 1600	1 : 3200
6–<7	5	0	2	3	0	0
7–<9	7	1	0	2	3	1
9–<11	8	0	4	3	0	1
11–<13	13	0	3	5	5	0
13–	8	1	5	0	2	0
Total	41	2	14	13	10	2

AKR)F_1 hybrids except for those kept on a separate rack, which were C3Hf, C57BL, BALB/c, and DBA.

Of 98 treated and untreated mice tested, 70 (71%) had polyoma-virus HI positive sera as shown in table 3. All 5 tested mice receiving injections of various tissue-culture fluids and bearing induced parotid tumors showed high HI titers (1:1600 to 1:12,800). Their inoculated but nontumorous cage mates were also 100 percent positive, though the titers were lower than those with parotid tumors. Of 22 mice receiving injections of fluids from control tissue cultures and of 20 untreated mice kept on the same rack with treated mice, none developed parotid tumors, but 16 (73%) and 17 (85%), respectively, had positive sera. Of 15 untreated mice 6 months of age or older, on a separate rack, 5 (33%) had positive sera, and of 6 mice younger than 6 months of age, none showed positive titers.

TABLE 3.—Frequency of positive polyoma-virus HI titers in treated and untreated mice of colony B

Group	Age in months	Number positive / Number tested mice	Percent positive	HI titer—number of mice								
				<1:100	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12,800
Inoculated* mice with induced parotid tumors	8–10	5/5	100						1		2	2
Inoculated* but non-tumorous cage mates of above group	9–12	12/12	100			1	4	2	4	1		
Controls†	8–14	16/22	73	2	4	1	7	5	3			
Untreated healthy mice on same rack	7–9	17/20	85	1	2	4	5	5	3			
Untreated healthy mice on separate rack	≧6	5/15	33	7	3	1	2	2				
Untreated healthy mice on separate rack	<6	0/6	0	6								
Leukemic mice‡	8–14	15/18	83	2	1	7	6	1	1			
Total		70/98	71									

*Receiving injections of inoculated tissue-culture fluids.
†Receiving injections of noninoculated tissue-culture fluids.
‡Inoculated and noninoculated.

The mice with leukemia, which occurred among treated or nontreated groups kept on the same rack as parotid-tumor-bearing mice, showed a percentage and pattern of HI titers similar to that of other mice older than 6 months of age without parotid tumors.

Frequency of Positive Polyoma-Virus HI Titers in Mice of Colony A Bearing Transplanted Tumors

For the investigation of the possibility of polyoma-virus propagation in transplantable tumors, mice grafted with 25 different tumors carried in colony A were tested for polyoma-virus HI titer. These tumors were 1 "sarcomatous type" tumor, 2 sarcomas, 4 lymphosarcomas, 6 mammary cancers, 1 lung tumor, 1 kidney tumor, 1 "abdominal" tumor, 1 uterine tumor, 1 testicular tumor, 2 ovarian tumors, 3 hepatomas, 1 pituitary tumor, and 1 plasma-cell leukemia. The results are shown in table 4.

TABLE 4.—Frequency of positive polyoma-virus HI titers in mice of colony A bearing any of 25 different transplanted tumors

	Months (birth to test)		Weeks (transplantation to test)			All ages combined
	<6	≧6	<3	3–6	>6	
Number positive / Number tested mice	36/113	13/40	7/22	22/52	20/79	49/153
Percent positive mice	32	33	32	42	25	32

Of 153 mice of all ages tested, 49 (32%) had HI positive sera. Of 40 mice older than 6 months of age, 13 (33%) had HI positive sera. In this transplanted tumor-bearing group, the mice younger than 6 months of age showed a positive rate of 32 percent (36 of 113), as compared with 0 percent of the non-tumor-bearing mice less than 6 months of age in this colony. The mice with tumors transplanted for a period of 3 to 6 weeks showed a 42 percent positive rate (22 of 52), and even those transplanted for less than 3 weeks showed a positive rate of 32 percent (7 of 22).

Recovery of Polyoma Virus From Transplanted Tumors

Table 5 shows the frequency of positive polyoma-virus HI titers in the mice of all ages listed in table 4 that had received transplants at least 3 weeks previously. Three weeks was selected as the shortest interval in which it could reasonably be expected that a high antibody titer might develop after exposure to polyoma virus. The only exception made was in the Gardner lymphosarcoma which (*a*) usually killed the host in less than 3 weeks, (*b*) produced some positive titers during the 3d week, and (*c*) from which polyoma virus was isolated.

In the first 8 tumors listed in table 5, no positive serology was found. For the next 9 tumors a low or moderate incidence of positive serology was found (8 to 33%). Five of the 9 were slightly higher than the 19 percent "background" level of untreated mice in colony A. An attempt to

recover polyoma virus from 7 of these 17 tumors by the tissue-culture technique gave a negative result.

For the remaining 8 tumors an incidence of positive serology from 40 to 100 percent was found. An attempt to isolate polyoma virus from 7 of these tumors by the tissue-culture technique gave a positive result for 5 tumors. These were the Gardner lymphosarcoma and a uterine sarcoma in the CBA strain, a mammary cancer in the BALB/c strain, a mediastinal lymphosarcoma in the DBAf strain, and a mesenteric lymphosarcoma in the C3H (Bittner) strain. The remaining tumor, a

TABLE 5.—Frequency of positive polyoma-virus HI titers in mice bearing tumor transplants for 3 weeks or longer in colony A

Tumor	Host strain or hybrid	Number positive	Number tested	Percent positive	Virus isolation
Mediastinal lymphosarcoma	DBAff	0	1	0	—
Abdominal sarcoma	A	0	5	0	—
Mammary cancer	A	0	6	0	—
Kidney tumor	BALB/c	0	8	0	
Ovarian tumor*	BALB/c	0	5	0	
Hepatoma*	C57BL	0	5	0	
Pituitary tumor	C57BL	0	1	0	
Plasma-cell leukemia*	C58	0	4	0	
Mammary cancer	DBAf	1	12	8	
Mammary cancer	DBAf	1	7	14	
Mammary cancer	BALB/c	1	6	17	—
Lung tumor	BALB/c	1	6	17	
Hepatoma*	NH	1	4	25	
Abdominal tumor	A	2	6	33	—
Testicular tumor	CE	2	6	33	—
Ovarian tumor*	NH	1	3	33	
Hepatoma*	DBAf	1	3	33	—
Sarcomatous-type tumor	(RIII × NH)F_1	4	10	40	—
Mammary cancer	BALB/c	3	6	50	—
Gardner lymphosarcoma†	CBA	2	3	67	+
Uterine tumor	STOLI	4	6	67	not tested (discontinued)
Mammary cancer	BALB/c	6	7	86	+
Mediastinal lymphosarcoma	DBAf	3	3	100	+
Uterine sarcoma	CBA	6	6	100	+
Mesenteric lymphosarcoma	C3H	5	5	100	+

*No tumor mass visible at time of bleeding.
†Tested in 2 to 3 weeks because this tumor usually kills hosts in less than 3 weeks.

uterine tumor, was discontinued before it was possible to test it by inoculation into tissue culture. Of the 22 HI-positive mice bearing transplants of the 5 tumors from which polyoma virus was isolated, the HI titers ranged as follows: 5 mice 1:200; 11 mice 1:400; 3 mice 1:800; 2 mice 1:1600; 1 mouse 1:3200.

Of these 8 tumors which provoked very high frequencies of positive polyoma-virus HI titers in host mice, 3 were "spontaneous" tumors (2 mammary cancers in the BALB/c strain and a uterine tumor in the STOLI strain). The others had developed in mice treated with various

combinations of castration, adrenalectomy, X ray, and methylcholanthrene.

Those tumors from which the virus was recovered usually induced polyoma-virus HI antibodies in host mice 2 to 4 weeks after their transplantation. However, a mammary cancer in the BALB/c strain and the Gardner lymphosarcoma in the CBA strain sometimes killed the host mice in less than 3 to 4 weeks, and before showing the development of noticeable HI antibodies.

DISCUSSION

The results of this survey essentially agree with those reported by Rowe *et al.* (*2*, *4*) and by Murphy and West (*3*). Mice with induced parotid tumors all showed high HI titers, whereas mice with leukemia were either positive or negative. When positive, their titers were generally lower and comparable to the positive titers observed among untreated controls. The polyoma virus is apparently transmissible from infected mice to other mice in close proximity as indicated by (*a*) the higher percentage of positive titers among untreated mice of colony B where more intensive work with induced parotid tumors was performed than in colony A; and (*b*) the higher percentage of positive titers among untreated mice on the same rack with mice bearing parotid tumors as compared to untreated mice on separate racks in colony B.

With respect to the 19 percent incidence of positive titers in colony A, it is uncertain to what extent this is related to the more recent work with leukemic-tissue filtrates, to what extent it results from the 5 transplanted tumor lines found to harbor and possibly disseminate polyoma virus, and to what extent it preceded either of these factors. Rowe *et al.* (*2*) have reported positive HI titers from colonies of mice in which no work with filtrate-induced tumors has been done, and from wild mice trapped in New York City and in rural Maryland (*4*).

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Regression of Grafted Bone Marrow in Homologous Irradiated Mouse Chimeras [1]

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SUMMARY

Lethally irradiated (C57BL × 101)F_1 mice receiving injections of C57BL bone marrow exhibited only a transitory production of graft-derived erythrocytes, the tendency for regression of the grafted marrow apparently varying with the X-ray dose and number of marrow cells infused. Judged on the basis of circulating erythrocytes, the grafted marrow persisted longer and regressions occurred later and less frequently in lethally than in midlethally irradiated recipients of 75 × 10^6 cells. Regression of grafted 101 marrow was not observed in lethally irradiated (C57BL × 101)F_1 mice, though such marrow did regress in midlethally irradiated hybrid recipients. The delayed mortality of the irradiated recipients was not consistently correlated with persistence or regression of the grafted marrow. The regression of grafted parental marrow noted under these conditions cannot as yet be conclusively explained, but might have resulted from an immunological rejection of the grafted cells. Genetic mechanisms for such a phenomenon are discussed, along with other mechanisms that might favor the selective growth of autologous hematopoietic cells.—J. Nat. Cancer Inst. 26:629–640, 1961.

STUDIES ON the survival of rat-mouse chimeras and on regression of grafted rat bone marrow in such mice in relation to the dose of X rays and of rat bone marrow cells have been reported (*1–3*). In these studies, the success or failure of transplantation was determined by serological and histochemical differences between cells of the donor and recipient. Factors that precipitate regression of grafted rat bone marrow in lethally irradiated mice are as yet incompletely defined, but the genetics of transplantation is less understood in a heterologous than in a homologous system, which, therefore, was chosen for two purposes: to ascertain whether survival of bone marrow chimeras is correlated with regression or persistence of the grafted marrow, and to study the influence of the dose of X rays and number of bone marrow cells injected.

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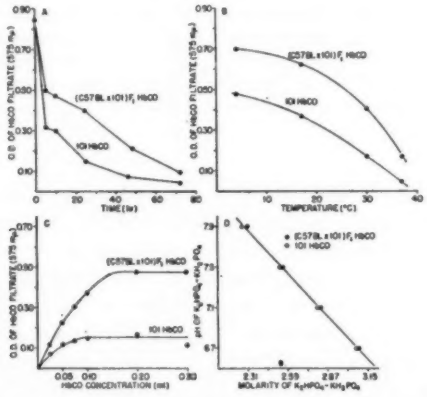
[3] Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

Parent-to-F_1 and F_1-to-parent systems were selected, since the F_1 hybrid theoretically should not produce antibodies against antigens of either parent, whereas parental tissues may produce antibodies against antigens of the F_1 hybrid (*4–6*). However, in all the combinations studied, bone marrow cells of the two parental strains differ in their killing effect on irradiated F_1 recipients (*5*, *7*). Experimental conditions were altered to promote good survival with persistence of the graft, poor survival with graft regression, or other combinations of these effects. In each case, the rate of appearance of circulating donor erythrocytes, survival of irradiated recipients, and status of the infused marrow graft were studied.

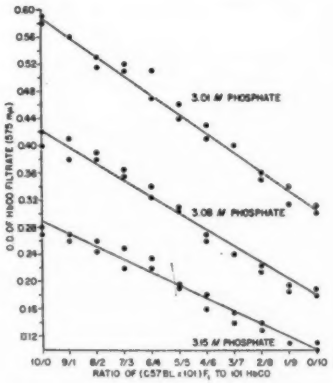
MATERIALS AND METHODS

Mice of the 101, C57BL, and (C57BL × 101)F_1 strains were used in this investigation. Four- to 6-month-old mice of each strain were used both as donors and recipients. Recipients were given midlethal, lethal, and supralethal doses of X rays. Thus, strain 101 mice were given 750, 850, and 950 r; C57BL mice, 600, 750, and 850 r; and (C57BL × 101)F_1 mice, 785, 900, and 1025 r. The dose rate of X rays was approximately 85 r/minute; mice were irradiated in revolving, lucite cages 93.7 cm from the source. Physical conditions were: 250 kvp, 31.5 ma, 0.1 mm of Al inherent filtration and 3 mm of Al added filtration, and HVL, 0.44 mm of

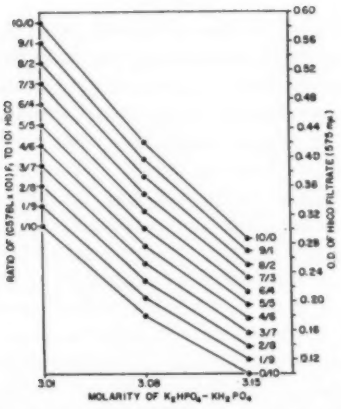


TEXT-FIGURE 1.—Effect of physical conditions on HbCO solubility. *A*. Effect of time: Preparations contained 0.10 ml of a 12 percent solution of HbCO, 0.50 ml of distilled water, and 4.4 ml of 3.5 M KH_2PO_4-K_2HPO_4 buffered at *p*H 6.7, 30° C. *B*. Effect of temperature: Optical density readings made at 21 hours of incubation; other physical conditions are listed above. *C*. Effect of HbCO concentration: Solubility of HbCO at various concentrations is shown; the plot shows that the system is saturated when 0.15 ml or more of a 12 percent solution of HbCO is used. *D*. Effect of *p*H: The molarities of phosphate buffer that yield equal optical-density readings at 4 levels of *p*H are plotted against the *p*H of the buffers. Optical density of the HbCO filtrates is inversely proportional to the *p*H of the buffer over the range examined.

Cu. Bone marrow, in quantities of 15×10^6, 30×10^6, or 75×10^6 nucleated cells, was injected intravenously 18 to 24 hours after X radiation, at which time the depression of the immune response of irradiated mice is greatest (*8*). Experiments were designed to avoid the Eichwald-Silmser effect (*9*) by the use of female C57BL and 101 mice as sources of donor marrow and female F_1 mice as recipients. The percentage of donor-type erythrocytes in irradiated recipients was determined on the basis of solubility differences in carbonmonoxyhemoglobin (HbCO) between donor and recipient (*10*). As previously reported, solubility of HbCO is affected by changes in *p*H, salt concentration, temperature, hemoglobin concentration, and incubation time (*10*). Effects of such conditions are presented (text-figs. 1, 2, and 3) to emphasize the importance of keeping the physical conditions under rigid control. Reproducibility of the results obtained by this method was considerably improved by an increase in the temperature and *p*H of the incubation mixture, and nomographs were constructed and used to correct for uncontrolled variables. The most satisfactory conditions for distinguishing the mouse hemoglobins in phosphate buffer were: 21 hours of incubation (30° C), 0.15 ml of whole blood, and 3.08 M KH_2PO_4-K_2HPO_4 buffered at *p*H 6.7. Small variations in incubation time, salt concentration, and *p*H were corrected by use of a correction graph (text-fig. 3). Under these conditions, the accuracy has been improved so that differences of ± 5 percent in the red cell population can be measured with reliability.


3.01 M PHOSPHATE
3.08 M PHOSPHATE
3.15 M PHOSPHATE
O.D. OF HbCO FILTRATE (575 mμ)
RATIO OF (C57BL x 101)F1 TO 101 HbCO
10/0 9/1 8/2 7/3 6/4 5/5 4/6 3/7 2/8 1/9 0/10

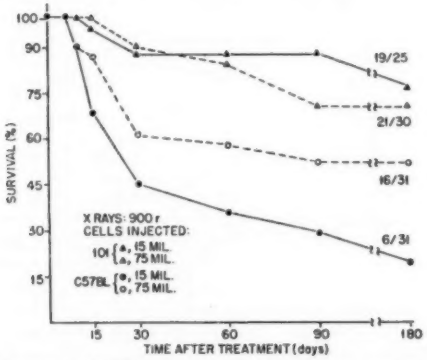
TEXT-FIGURE 2.—Solubility of mixtures of (C57BL × 101)F_1 and 101 HbCO. The optical density of the HbCO filtrates is plotted for mixtures of (C57BL × 101)F_1 and 101 HbCO in 3.01, 3.08, and 3.15 M phosphate under optimal conditions.



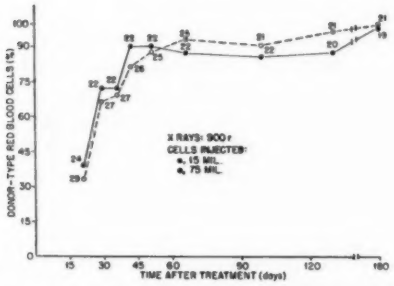
TEXT-FIGURE 3.—Relation of the ratio of (C57BL × 101)F_1/101 erythrocytes to HbCO solubility. Optical densities for each mixture of (C57BL × 101)F_1 and 101 HbCO (text-fig. 2) are plotted for the 3 molarities of phosphate used. Points of equal ratios in 3.01, 3.08, and 3.15 M phosphate are connected. Although factors of time, temperature, Hb concentration, *p*H, and salt concentration can be maintained moderately constant, small deviations from optimal conditions were usually obtained in daily experiments as a result of even seemingly insignificant deviations from each variable. It was experimentally determined that such variations could be corrected, at least to a large extent, by use of this nomograph; *i.e.*, if (C57BL × 101)F_1 and 101 HbCO filtrates of control mice read 0.38 and 0.16, respectively, rather than readings of 0.42 and 0.18, an OD value of 0.225 for that day's experiment, read between the control points, indicates that 70, not 80 percent, of the erythrocytes are of donor type. Similar correction graphs were made for other combinations studied.

RESULTS

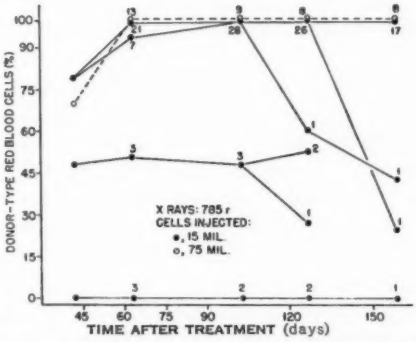
Lethally irradiated F_1 mice given 101 bone marrow survived equally well whether receiving injections of 15×10^6 or 75×10^6 nucleated cells (text-fig. 4) and showed nearly complete conversion of red blood cells to donor type within 60 days, with no subsequent reversions to F_1 type (text-



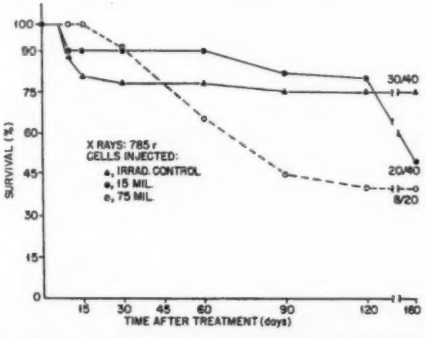
TEXT-FIGURE 4.—Survival of 900 r irradiated (C57BL × 110)F_1 mice receiving cells of 101 or C57BL parent. Number of survivors/number of recipients at 180 days is indicated for each group.



TEXT-FIGURE 5.—Circulating 101 donor erythrocytes in 900 r irradiated (C57BL × 101)F_1 recipient mice. The points represent an average value for each group; deviations were usually less than ±8 percent; thus they were plotted as a single point. Numbers next to each point on the graph represent the number of animals examined.



TEXT-FIGURE 6.—Circulating 101 donor erythrocytes in 785 r irradiated (C57BL × 101)F_1 recipient mice. Incomplete conversion to donor-type red cells and reversions to host-type red cells are indicated. In general, results are plotted in a single point when the percentages of donor-type erythrocytes differed by less than ±5 percent. The incidence of deaths is indicated by a decrease in the number of animals examined at each point; the line is discontinued when all the animals of a group died.

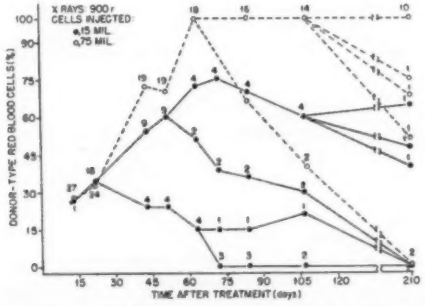


TEXT-FIGURE 7.—Survival of 785 r irradiated (C57BL × 101)F_1 mice receiving cells of 101 parent. For explanation *see* legend to text-figure 4.

fig. 5). At midlethal doses of X rays a few reversions to F_1 type were noted, but only in the group receiving the smaller cell inoculum (text-fig. 6); moreover, the survival of midlethally irradiated F_1 recipients of 101 bone marrow cells was inferior to that of the controls (text-fig. 7).

When C57BL bone marrow was infused into 900 r irradiated F_1 recipients, the 30-day survival was slightly better if 75×10^6 cells were injected than if 15×10^6 cells were used (text-fig. 4), and the incidence of late deaths (10 vs. 26%) was greater in the latter. Although marrow repopulation was completed at 60 days after inoculation of 75×10^6 nucleated cells of C57BL marrow, reversion had started by 90 days and was completed by 210 days in 13 percent of the mice (text-fig. 8). In 20 percent of the mice, reversion started later and was only partially completed by 210 days. In contrast to results after larger cell inoculum, marrow repopulation was incomplete after the inoculation of 15×10^6 cells. At 105 days 77 percent of the recipients were still partial chimeras, while 22 percent represented reversals (text-fig. 8). One F_1 reverted even after receiving 1025 r and 15×10^6 cells of C57BL marrow (table 1). Additional data on the frequency of reversions in F_1 mice at 180 days after receiving various doses of X rays and cell inoculum are presented in table 1. Data in text-figures 5, 6, and 8 are included for comparison.

When (C57BL $\times$ 101)F_1 bone marrow was infused into 850 r irradiated strain 101 mice, 10 of 12 transplants persisted after 75×10^6 cells were injected, but all grafts rapidly regressed in such mice infused with only 15×10^6 F_1 cells (text-fig. 9). F_1 bone marrow grafts did not regress in supralethally (950 r) irradiated 101 recipients even when as few as 15×10^6 cells were injected, but reversions were observed even after infusion of the larger number of cells in midlethally irradiated 101 mice (table 2). Similarly, reversions to host type occurred when F_1 cells were injected into C57BL recipients given a midlethal (600 r) dose of X rays, but grafted F_1 cells persisted in such recipients given a lethal (750 r) or supralethal (850 r) dose of X rays (table 2). Sixty-seven of the parental strain recipients that possessed grafted F_1 cells survived more than 30 days; of these, 47 survived 180 days or more after treatment. Of the latter group, 74 percent had erythrocytes of F_1 type only (table 2).



TEXT-FIGURE 8.—Circulating C57BL donor erythrocytes in 900 r irradiated (C57BL $\times$ 101)F_1 recipient mice. For explanation *see* legends to text-figures 5 and 6.

TABLE 1.—Frequency of reversions in (C57BL × 101)F_1 mice at 180 days after receiving a supralethal, lethal, or midlethal dose of X rays and an inoculum of 15, 30, or 75 × 10^6 parental bone marrow cells

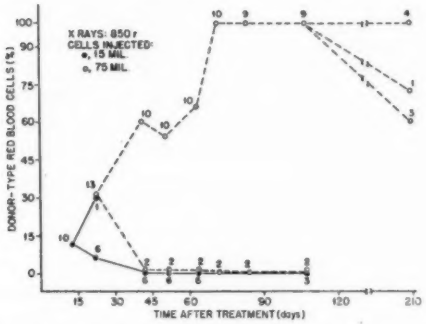
Donor		Recipient					
Strain	Number of nucleated cells (× 10^6)	Dose of X rays (r)	Number of mice receiving injections	Number of survivors at 180 days	Reversals* (%)	Partial* chimeras (%)	Repopulated* (%)
101	30	950	98	65	0	0	100
	15	900	25	19	0	0	100
	75	900	31	21	0	0	100
	15	785	40	17	12	47	41
	75	785	20	8	0	50	50
C57BL	15	1025	22	8	0	12	88
	75	1025	13	2	0	0	100
	30	950	139	50	0	20	80
	15	900	51	16	44	50	6
	75	900	31	15	13	20	67
	15	785	19	11	45	55	0
	75	785	12	4	0	75	25

*Recipients with less than 10 percent donor-type red cells are considered reversals; chimeras possessing 10–89 percent donor-type red cells are classed as partial chimeras; and chimeras with 90–100 percent donor-type red cells are considered repopulated. Percentage of the recipients in each class is calculated in terms of the number of animals/number of survivors at 180 days.

TABLE 2.—Frequency of reversions in 101 and C57BL mice at 180 days after receiving a supralethal, lethal, or midlethal dose of X rays and an inoculum of 15, 30, or 75 × 10^4 (C57BL × 101)F_1 bone marrow cells

Donor		Recipient					
Number of nucleated (C57BL × 101)F_1 cells (× 10^4) injected	Strain	Dose of X rays (r)	Number of mice receiving injections	Number of survivors at 180 days	Reversals* (%)	Partial* chimeras (%)	Repopulated* (%)
15	101	950	20	9	0	0	100
75		950	9	3	0	0	100
15		850	30	0†	—	—	—
75		850	30	8	0	50	50
30		750	20	2	100	0	0
75		750	10	1	0	0	100
30	C57BL	850	10	8	0	0	100
75		850	15	4	0	0	100
30		750	10	1	0	0	100
75		750	10	5	0	0	100
30		600	10	6	100	0	0
75		600	10	0†	—	—	—

*For explanation, *see* legend in table 1.
†In each of these groups, 6 recipients were alive at 60 days after treatment and all showed reversal; however, they died before the 180-day period.



TEXT-FIGURE 9.—Circulating (C57BL × 101)F_1 donor erythrocytes in 850 r irradiated 101 recipient mice. For explanation, *see* legends to text-figures 5 and 6.

DISCUSSION

Delayed disappearance of graft-derived, parental-type erythrocytes noted in F_1 recipients suggests that the infused marrow graft is continuously competing with the few autologous cells that survive X radiation. Supporting evidence for such competition was found in F_1 recipients that received an infusion of 75×10^4 isologous marrow cells along with 15×10^6 cells of C57BL marrow; in such recipients a complete reversion to F_1 type occurred within 180 days after an initial, partial blood chimerism ($\sim$ 80% red cells of C57BL type). The frequency of reversions was a function of the X-ray dose and number of bone marrow cells infused in that reversions were more frequent when the marrow graft was incompletely established as a result of insufficient irradiation or inoculum. The growth of the autologous cells might have been favored because of immunological or physiological reasons, among other possibilities.

In view of previous demonstrations of reversions of chimera blood to recipient type in heterologous chimeras (*1*, *2*), the possibility that reversions in our experiments might be immunological in nature must be considered. Although this seems unlikely, since it is usually assumed that all parental antigens are expressed in the F_1, such may not necessarily be the case (*11*).

Immunological regression of parental marrow in irradiated F_1 recipients might occur if parental antigens are lacking in the F_1. Mechanisms for such a phenomenon may be as follows: (*a*) Some antigens produced in the parent may be governed by recessive genes and thus may not be expressed in the F_1; (*b*) hybrid substances may be formed in the F_1 that are antigenically dissimilar to their parental counterparts, and thus cause the F_1 to lack parental antigens—hybrid substances have not been demonstrated in the mouse, but such substances have been recognized in other species (*12*, *13*); (*c*) interaction of alleles at independent loci may result in formation of antigens in the parent that may not be expressed in the F_1 (*14*).

These experiments were designed to avoid the sex-linked histoincompatibility effect noted by Eichwald and Silmser (*9*) through the use of females both as donors and recipients; however, if males had been used both as donors and recipients, which seemingly avoids the Eichwald-Silmser effect in (C57BL × CBA)F_1 mice (*15*), a weak F_1 against parent reaction might have occurred if the sex chromosomes of parental strains had borne different histocompatibility loci. By use of skin grafting, Congdon has observed some heterogeneity among commercially produced strain 101 mice used in this experiment, but not among strain C57BL mice, exclusive of the Eichwald-Silmser effect (personal communication). Such heterogeneity might cause regression of 101 marrow in (C57BL × 101)F_1 mice; however, it fails to explain the observed regression of C57BL marrow in F_1 mice.

Another possible explanation for regression of parental bone marrow in irradiated F_1 recipients might be the existence of physiological, nutritional, or other factors favoring the growth of the autologous marrow cells (*16*). The reversion of F_1 recipients that received injections of a few isologous cells along with many C57BL cells, mentioned previously, is consistent with the idea that there is a micro-environmental advantage for the host cells, albeit such an advantage may in part stem from immunological factors.

However, that such reversions may not necessarily be of an immunological nature was suggested by the observation that the marrow and spleens of 4 lethally irradiated (C57BL × 101)F_1 mice that seemed to have reverted completely to F_1 type, after earlier repopulation by C57BL cells, nevertheless contained some latent C57BL stem cells. Three months after the reversions, bone marrow and spleens were removed from the 4 seemingly completely reverted primary F_1 recipients of C57BL bone marrow and were infused into 10 secondary X-irradiated (C57BL × 101)F_1 recipients; C57BL red cells later appeared in the peripheral blood of 8 of the 10 secondary X-irradiated (C57BL × 101)F_1 recipients, comprising 10 to 40 percent of their circulating erythrocytes 30 to 70 days post treatment (Popp and Cosgrove, unpublished data). The presence of latent donor-type cells in reverted (C57BL × 101)F_1 mice suggests that such grafted cells grow less well than autologous cells; furthermore, they indicate that, although detection of donor-type cells is proof of chimerism, absence of donor-type cells in the peripheral blood does not prove that the blood-forming tissue no longer has latent donor-type cells. On the other hand, these observations do not rule out a possible immunological nature for the regression of parental grafts, since the parental stem cell, being immature, may not possess all the antigens of its more differentiated forms, and thus may be antigenically less distinguishable from the host's own stem cell.

In the evaluation of the reversions and survival patterns of these experiments, it was assumed that the type of erythrocyte found in the peripheral blood indicated both the hematopoietic and immunological status of the irradiated recipients, since Vos *et al.* (*17*) did find matching

red blood cell and lymphocyte serotypes in the bone marrow and lymph nodes of homologous chimeras. Our data show many (C57BL × 101)F_1 mice that died 30 to 180 days after injection of parental bone marrow did not express functional grafts (text-figs. 6 and 8); these deaths, though late, need not be ascribed to a graft-anti-host reaction but may have been caused by other pathological conditions such as those discussed by van Bekkum *et al.* (*18*). However, the reverted red cell chimeras may have been chimeras with regard to their white blood cells and their deaths may have resulted from a graft-anti-host reaction.

The observation that grafts persisted when a larger number of cells was injected, whereas temporary transplants resulted when a smaller number of bone marrow cells was administered (text-fig. 9), suggests that one might reduce the X-ray dose to recipients and yet obtain good transplants by increasing the number of bone marrow cells injected; a larger number of cells may partially inhibit or swamp the residual immunological response of the host (*2*). A deleterious effect at midlethal X-ray doses has been observed with rat-mouse chimeras (*18*) and a comparison of text-figures 4 and 7 suggests that similar effects may also occur in homologous chimeras. Moreover, reversions reported here when F_1 bone marrow cells were injected into midlethally irradiated parental strain mice may be similar to graft regressions observed in a human radiation accident. At least 30×10^6 F_1 bone marrow cells were required for good transplantation in parental strain mice given an LD100/30-day-dose of X rays. This number of cells per 25 g mouse, based on number of cells per g body weight, suggests that 10^{11} cells or more may be required for homologous bone marrow therapy in humans; whereas only approximately 10^{10} cells have been administered to human patients to date (*19*).

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Immunogenetic Studies of a Histocompatibility Hemagglutinogen (R-1) in the Rat [1, 2]

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SUMMARY

These immunogenetic studies in the rat establish the control of the R-1 factor hemagglutinogen B by a dominant gene following simple Mendelian laws and show its relationship to the inheritance of susceptibility to tumor-tissue implantation. Rats segregating as R-1 negative in the F_2 and backcross generations were not only able to resist R-1 positive tumor implantation, but immunization induced the specific anti-R-1 (B) hemagglutinin and its associated cytotoxic antibody. In contrast to the *H-2* system in the mouse, the R-1 factor hemagglutinogen B is found not only in embryonic tissues but in the erythrocytes of R-1 positive rats of all ages. In addition, 17 rat strains were screened for the B hemagglutinogen. Six *inbred* rat strains were positive and 9 were negative. The *randombred* Wistar strain (90% R-1 positive) and the *penbred* Chocolate strain (88% R-1 positive) illustrate the heterogeneity expected of noninbred systems. Eight transplantable rat tumors were tested; 5 tumors contained the R-1 factor hemagglutinogen B.—J. Nat. Cancer Inst. 26: 641–649, 1961.

GORER'S DISCOVERY of antigen II in the *mouse* (*1*, *2*) led to extensive studies of the *H-2* hemagglutinogen and to the elucidation of the role of histocompatibility antigens in homograft survival (*3–7*). This extension of the genetic theory of tumor transplantation advanced by Little (*8*) and Little and Strong (*9*) did much to establish the use of inbred mouse strains in oncology.

On the other hand, the rat, a species gaining in importance as an experimental tool in transplantation studies, has been subject to the erroneous view that it could not be inbred to genetic homogeneity. Billingham and Silvers (*10*) point out this misconception and have

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negated it by the results of their reciprocal skin-homografting studies of several rat strains.

The possibility of the existence of a histocompatibility hemagglutinogen in the rat was indicated by the early work of Lumsden, using the Jensen rat sarcoma (*11*). Recently, the authors (*12*), using several strains of rats and 2 transplantable rat ascites tumors, were able to work up the R-1 factor hemagglutinogen B, a major histocompatibility antigen in the rat, whose serologic activity parallels, in many respects, the serologic activity of the *H-2* hemagglutinogen in the mouse. The subject of this report, therefore, is the results of immunogenetic studies establishing the control of this factor by a dominant gene and its relationship to the inheritance of susceptibility or resistance to tumor-tissue implantation. In addition, a number of rat strains, as well as several transplantable rat tumors, have been screened for the R-1 hemagglutinogen B to determine their R-1 status.

MATERIALS AND METHODS

Rat strains.—The P.A. strain of rats used in these studies was originally inbred (brother × sister) for 135 generations as the King Albino by Helen Dean King, and further inbred (brother × sister) for 21 generations in the Aptekman Colony of the Wistar Institute and now for over 4 generations in the Bogden Colony of the Biochemical Research Foundation. The P.A. rat has never been bred commercially, so that only a small colony of a carefully controlled, single-trunk, inbred nucleus has been maintained.

The Lewis strain of rats, originally from the randombred Wistar Institute strain, had been inbred (brother × sister) for 20 generations by Margaret Reed Lewis, and further inbred for 10 generations (brother × sister) in the Aptekman Colony of the Wistar Institute. This strain, too, had never been bred on a large scale, but had been maintained as a single-trunk, inbred nucleus.

The homogeneity of the inbred P.A. and Lewis strains has been verified by the sensitive method of reciprocal skin homografting of rats taken at random (*10*). The homogeneity of the P.A. strain for the R-1 factor hemagglutinogen B, as well as for the *lack* of the same factor in the Lewis strain, has been indicated in previous studies (*12*).

Wistar strain rats were received from the commercial colony of the Wistar Institute. Although quite homogeneous as concerns their physical morphology, this strain has always been carefully randombred. In addition to variation in the susceptibility of the Wistar strain rats to their "native" tumor (*12, 13*), the heterogeneity of the strain has been further indicated by the results of reciprocal skin homografting of rats taken at random (*10*) and the results of screening for the R-1 factor hemagglutinogen B (*12*).

The Chocolate strain, penbred in our animal colony for 8 years, was

received from Doctor Helen Dean King as mutants from the Wild Grey Norway rat.

The inbred Copenhagen, Selfed, August, and Zimmerman rat strains were received from Doctor W. F. Dunning, and the inbred Albany, Buffalo, A × C, Fischer, Marshall, O-M/N, Wistar, CA/R, and CA/S rat strains were received from Doctor Donald W. Bailey.

Ascites tumors.—The ascites tumor, designated as AA, originated spontaneously in a female breeder of the Wistar Institute strain of rats. It was transplanted from the original host and has been carried by serial intraperitoneal passage of the ascites only in rats of the strain of origin. Its transplantation and growth characteristics have been reported elsewhere (*13*).

The ascites tumor, designated as 9A, originated spontaneously in a female breeder of the inbred P.A. strain of rats. It was transplanted from the original host and has been carried by serial, intraperitoneal passage of the ascites only in rats of the strain of origin. The routine transplantation inoculum of 0.1 ml (4- to 5-day-old tumor), always lethal intraperitoneally, produces no lethal tumors subcutaneously in the P.A. strain. Challenge inoculums for both tumors were 0.1 ml ascites of a 3- or 4-day-old tumor.

Preparation of serums and hemagglutination tests.—These procedures have been described in detail in a previous publication (*12*).

RESULTS

Inheritance of the R-1 Factor Hemagglutinogen B

Results of screening the inbred P.A. and Lewis strains of rats for the R-1 factor hemagglutinogen B indicated the phenotypic homogeneity to be expected of inbred systems (*12*). Rats of the P.A. strain were all positive, in contrast to the complete lack of this hemagglutinogen in every rat of the Lewis strain tested.

Homogeneity for even very minor antigens was further indicated by the results of reciprocal skin homografts of rats taken at random in the 2 strains (*10*).

Because of these facts, it was decided to cross the P.A. and Lewis strains reciprocally to ascertain genetic control of the B antigen (R-1 factor hemagglutinogen B). The results of such a reciprocal cross, as well as the backcross of the F_1 with the parental generation, are shown in table 1.

It is quite evident that antigen B is controlled by a dominant gene and follows simple Mendelian laws. Furthermore, since the animals making up the parental generation were chosen at random from the 2 strains, it would appear that the P.A. strain is homozygous (BB) for the B antigen and the Lewis strain is homozygous (bb) for the lack of it.

All animals tested in the F_1 generation were found to be phenotypically positive for the hemagglutinogen (Bb), segregation of the gene determining

TABLE 1.—Inheritance of the R-1 hemagglutinogen: P.A. (R-1$^+$) × Lewis (R-1$^-$)

Generation	Number tested/ No. R-1$^+$	Ratio R-1$^+$:R-1$^-$ Theoretical	Actual
F_1	{25 ♂/25 ♂ 20 ♀/20 ♀	100% R-1$^+$	100% R-1$^+$
F_2	{26 ♂/18 ♂ 11 ♀/ 9 ♀	3:1	2.7:1
Backcross of F_1 with parental generation			
F_1 × P.A.	{31 ♂/31 ♂ 27 ♀/27 ♀	100% R-1$^+$	100% R-1$^+$
F_1 × Lewis	{28 ♂/19 ♂ 11 ♀/ 7 ♀	1:1	1.5:1

the presence of hemagglutinogen B occurring in the F_2 generation with the actual ratio of R-1$^+$ to R-1$^-$ (2.7:1) in excellent agreement with the theoretical ratio (3:1).

In the backcross of the F_1 (Bb) with the P.A. strain parental generation (BB), all animals were phenotypically positive, with segregation of the gene determining the presence of hemagglutinogen B occurring only in the backcross of the F_1 (Bb) with the Lewis strain parental generation (bb). Here, too, the actual ratio of 1.5:1 closely approximated the expected theoretical ratio of 1:1.

Genic dominance in simple Mendelian inheritance of the B hemagglutinogen is further substantiated by the following study: An R-1 negative male and female were chosen at random from the carefully randombred Wistar strain of rats. This R-1 negative subline was desiguated "RN" and the careful randombreeding continued for over 4 generations. Of over 150 RN subline rats tested in the various generations, none have been positive for the B hemagglutinogen. It would appear, therefore, that a rat found to be B negative is homozygous negative for the factor.

Inheritance of Susceptibility to Tumor Implantation

Previous transplantation studies of the AA tumor in its native Wistar strain rats pointed out the parallelism between susceptibility (90% lethal takes) and the presence of the R-1 factor hemagglutinogen B in 90 percent of the Wistar strain (*12*).

To ascertain whether inheritance of the B antigen was paralleled by susceptibility to tumor implantation, reciprocal crosses were made with rats of the B hemagglutinogen-positive P.A. strain and the hemagglutinogen-negative Lewis strain. After determination of the R-1 status of the animals in the various generations (table 1), they were allowed to mature to 60 days of age and then implanted intraperitoneally with 9A ascites to determine their susceptibility to tumor implantation, or they were allowed to mature further and were used as breeders. Having served their function as breeders, these animals were also tested for susceptibility to 9A ascites implantation. It should be pointed out that the 9A

TABLE 2.—Inheritance of susceptibility to R-1 positive tumor grafts*: P.A. strain (R-1$^+$) × Lewis strain (R-1$^-$)

Generation	R-1 positive rats		R-1 negative rats	
	Number implanted/ number dead	Percent susceptible	Number implanted/ number dead	Percent susceptible
F_1	25♂/25♂ 20♀/20♀	100	—	—
F_2	18♂/18♂ 10♀/10♀	100	7♂/1♂ 2♀/0♀	11
Backcross of F_1 with parental generation				
F_1 × P.A.	31♂/26♂ 27♀/22♀	83	—	—
F_1 × Lewis	18♂/17♂ 6♀/5♀	92	9♂/1♂ 6♀/1♀	13

* Susceptibility indicates death due to progressive tumor growth; 9A ascites tumor native to the P.A. strain.

ascites tumor is native to the P.A. strain and contains the B hemagglutinogen. The results of these implantations are summarized in table 2.

Of the 73 R-1 positive animals implanted in the F_1 and F_2 generations, 100 percent died from progressive tumor growth. Only 85 percent of the 82 R-1 positive backcross animals implanted died as a result of tumor growth.

On the other hand, 11 to 13 percent of the R-1 negative rats in the F_2 and backcross generations died from tumor implantation.

It is evident, therefore, that inheritance of susceptibility to implantation of tumor tissue containing the histocompatibility hemagglutinogen B (table 2) parallels very closely inheritance of this same factor (table 1).

Screening Rat Strains and Rat Tumors for the R-1 Hemagglutinogen B

In view of the genetic control of this factor and its role as a major histocompatibility antigen, a number of rat strains and transplantable rat tumors were screened for the presence or absence of the B hemagglutinogen.

We screened rat strains by testing their erythrocytes against anti-B serum. Tumors were tested for their ability to induce the anti-B hemagglutinin in B negative rats and to absorb the anti-B hemagglutinin *in vitro*.

Of 15 inbred rat strains screened, 6 were positive for the B hemagglutinogen and 9 were negative. The randombred Wistar strain (90% B positive) and the penbred Chocolate strain (88% B positive) emphasize the heterogeneity expected of noninbred systems (table 3).

Ten Sprague-Dawley strain rats purchased commercially were tested for the B antigen. Three rats were negative and 7 were positive. These results indicate that this strain is also heterogeneous for the B hemagglutinogen.

TABLE 3.—Results of screening for the R-1 factor hemagglutinogen (B) in the erythrocytes of several rat strains*

Strain	Line	Generation	Type of breeding	Number positive/ Number tested	Percent positive
P.A.		F_{183+}	Inbred	275/275	100
A × C	9935	F_{50+}	Inbred	10/10	100
O-M/N		F_{17+}	Inbred	10/10	100
Selfed	36670	F_{25+}	Inbred	6/6	100
Copenhagen	2331	F_{53+}	Inbred	6/6	100
August	7322	F_{62+}	Inbred	6/6	100
Lewis		F_{33+}	Inbred	0/110	0
Albany		F_{8+}	Inbred	0/10	0
Buffalo		F_{20+}	Inbred	0/10	0
Fischer	344	F_{59+}	Inbred	0/10	0
Marshall	520	F_{62+}	Inbred	0/10	0
Wistar		F_{23+}	Inbred	0/10	0
CA/R		F_{23+}	Inbred	0/10	0
CA/S		F_{29+}	Inbred	0/10	0
Zimmerman	61	F_{68+}	Inbred	0/6	0
Wistar			Randombred	247/275	90
Chocolate			Penbred	42/48	88

*Rats chosen at random.

TABLE 4.—Results of screening for the R-1 factor hemagglutinogen (B) in transplantable rat tumors

Tumor designation	Strain of origin	Type of tumor	R-1 status
AA	Wistar (randombred)	Ascites	+
9A	P.A.	Ascites	+
Yoshida sarcoma	Japanese (noninbred)	Ascites	−
LK2 leukemia	P.A.	Lymphocytic	+
Walker carcinosarcoma 256	Walker*	Solid	−
Lymphoma #8	Lewis	Solid (lymphoma)	−
Carcinoma #5	P.A.	Solid	+
Sarcoma #6	P.A.	Solid	+

*Rats from a commercial source bred in Dr. George Walker's colony (*14*) and personal communication, Dr. M. R. Lewis.

Of 8 transplantable rat tumors tested, 5 tumors contained the B hemagglutinogen and 3 were negative (table 4). Of the 5 tumors that were positive, 4 were from the P.A. strain known to be homozygous for the gene, and 1 was from the heterozygous Wistar strain.

Serologic Studies

In addition to the determination of the R-1 status and susceptibility to implantation of the 9A ascites tumor, R-1 negative rats in the various generations resulting from the P.A. and Lewis strain cross were further immunized by repeated inoculations (2 to 3) of 0.1 ml 9A ascites. The serum from such animals was tested for hemagglutinin (anti-B) and cytotoxic activity. Each of 20 animals tested in this manner was capable of responding to the antigenic stimulus of the R-1 positive 9A tumor by forming humoral antibodies with specific anti-B and cytotoxic activity.

The question of postnatal "antigenic maturation" of the B hemagglutinogen in the erythrocyte was resolved as follows: Aliquots of pooled, hyperimmune, Wistar anti-B serums were tested against washed erythrocytes prepared from blood drawn by heart puncture from rats of the P.A. strain 1 to 60 days of age.

Erythrocytes from rats of all ages were agglutinable by Wistar anti-B serum. However, hemagglutinin reactions with erythrocytes from rats over 30 days of age appeared somewhat stronger than with erythrocytes from younger animals. All reactions were definitely agglutination and not just strong rouleaux formation.

Furthermore, minced embryonic tissue, removed from a normal P.A. strain female in the 18th day of the gestation period, was capable of inducing the specific anti-B hemagglutinin in R-1 (B) negative rats.

DISCUSSION

The mouse, like the fruit fly, because of its size has certain advantages for genetic and immunogenetic studies. The rat also has many advantages for experimental study, and because of its larger size provides not only a greater yield of both normal and malignant tissue for study, but also of serum for electrophoretic and serologic testing, which thus provides the great advantage of allowing the investigator to carry out operative and immunologic procedures on the *individual* animal without the necessity of pooling or using tedious microtechniqes. Furthermore, rat serum has good complement activity, comparing favorably with guinea-pig complement in some hemolytic systems.

Kim, Clifton, and Furth (*15*) point out that the rat has not been used to full advantage in cancer research, in part because of inadequate knowledge of the spectrum of tumors and their character in the available inbred strains of rats, and in part because of the poor transplantability of rat tumors due to the heterozygosity of the rat strains used.

It is unfortunate that misinterpretations of early inbreeding and grafting experiments (*16*, *17*) led to the erroneous view that rats cannot be inbred to the point of genetic homogeneity. That this view is fallacious and that true antigenic homogeneity can be obtained in the rat were shown by the exhaustive grafting tests carried out by Billingham and Silvers (*10*). The results of their tests have shown that the inbred P.A. and Lewis rat strains were ". . . in no sense inferior to accredited isogenic mouse strains in respect to uniform long-term acceptance of intrastrain grafts."

That inbred strains of rats are now in existence and available is pointed out by the stocks of rats listed by Billingham and Silvers in an appendix to the report of their investigations (*10*).

That the rat may have a comparable system of histocompatibility antigens as has the mouse is further borne out by the results of our studies of the R-1 factor hemagglutinogen B (*12*). The appearance of the anti-B hemagglutinin in B-negative animals resistant to tumor implantation,

the distribution of the B hemagglutinogen in normal and neoplastic tissues, its association with a cytotoxic antibody, and the very definite parallelism between the activity of the R-1 antigens in the rat and the *H-2* antigens in the mouse would tend to support this belief.

The present study substantiates the control of this hemagglutinogen by a dominant gene and further indicates its function as a major histocompatibility antigen by the parallelism between the inheritance of the B antigen and the inheritance of susceptibility to B-positive tumor implantation (tables 1 and 2).

Homozygosity of inbred rat systems, as well as the distribution of the R-1 factor hemagglutinogen B in various rat strains, can be seen in table 3. It is evident that there are now available to the investigator inbred rat strains differing by an easily identifiable, major histocompatibility antigen.

Gorer (*2*) found that the erythrocytes of newborn *H-2*-positive mice were not agglutinable by the anti-*H-2* hemagglutinin. Mouse erythrocytes became agglutinable only at about 6 weeks of age. In our studies, erythrocytes of newborn R-1 positive rats were agglutinable by the anti-R-1 hemagglutinin, though the reactions were not quite as strong as with erythrocytes of rats 30 days of age or older. Furthermore, the presence of this antigen in embryonic tissue was indicated by the fact that minced embryonic tissues from a homozygous positive (BB) P.A. strain female were capable of inducing the specific anti-B hemagglutinin in B negative (bb) rats.

The genetic theory of tumor transplantation advanced by Little (*8, 9*), which has been consolidated and confirmed by many investigators (*18*), is summarized by Gorer (*2*) in his discussion of the genetic and antigenic basis of tumor transplantation. If a tumor arises in a member of a "pure" line, it will usually grow in 100 percent of cases when transplanted to other members of the same "pure" line. If two "pure" lines are crossed, one susceptible and the other resistant, the first generation hybrids (F_1) are all susceptible; similarly the progeny produced by backcrossing the F_1 to the susceptible "pure" line are also susceptible.

We also find that tumors arising in inbred rat strains are 100 percent transplantable, and the results of the present study (table 2) substantiate the susceptibility of the F_1 hybrid. However, we have found that the progeny resulting from backcrossing the F_1 to the susceptible P.A. strain were only 87 percent susceptible. It should be stressed at this point that the 9A ascites tumor, native to the P.A. strain, and 100 percent lethal when implanted intraperitoneally, has always lacked the ability to produce lethal subcutaneous tumors in the P.A. strain, and that regression of subcutaneous implants is followed by an immunity to subsequent intraperitoneal inoculums (*19*). Thus, a peculiar growth characteristic coupled with the possibility of subcutaneous seepage of the 9A ascites and an increased histoincompatibility resulting from the backcross may account for the absence of the 100 percent susceptibility expected.

Gorer (*2*) goes on to point out that the number of susceptible individuals resulting from the backcross to the resistant line and in the F_2 generation

varies with the tumor used. In some cases 50 percent of the former and 75 percent of the latter generations may be susceptible, while in others the number is much less. Here our data are in close agreement. The offspring resulting from the backcross with the resistant Lewis strain were 62 percent susceptible and the F_2 generation was 78 percent susceptible.

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Induction of Tumors in Newborn Mice With Dibenz[a,h]anthracene and 3-Methylcholanthrene [1]

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SUMMARY

Multiple tumors of the lung, subcutaneous fibrosarcomas, leukemias, sebaceous-gland adenomas, and hepatomas have been induced within 8 to 32 weeks after a single, subcutaneous injection or 0.06 mg dibenz[a,h]anthracene or 0.1 mg 3-methylcholanthrene into newborn mice (noninbred albino, C3H/P, and C57BL/6JN). The doses were well tolerated and acute toxicity was negligible. Direct comparison showed that newborn mice were more susceptible to induction of pulmonary tumors than older sucklings or adult mice. Newborn mice of the albino strain were most susceptible to lung-tumor induction, and newborn C57BL mice the least. Essentially 100 percent of albino mice treated during the first 18 hours after birth developed pulmonary tumors within 16 to 24 weeks after subcutaneous administration of 0.02 mg dibenz [a,h] anthracene or 0.011 mg 3-methylcholanthrene, and 38 percent developed pulmonary tumors after 0.0022 mg dibenz[a,h] anthracene. Repeated experiments have given consistent data on latent period, incidence of induced pulmonary tumors, and mean number of tumor nodules per animal. The latter measurement appears to be the more sensitive index of response and/or carcinogenic potency. Histopathologic study of these neoplasms suggested that the carcinogenic process in the lung was sustained and that new tumors were forming as late as 24 weeks after injection of carcinogen. Study of induced subcutaneous tumors showed that many arose from the perimysium of subcutaneous muscle, though they grew as spindle-cell fibrosarcomas. A greater variety of tumors was induced with 3-methylcholanthrene than with dibenz[a,h]anthracene. Mice given 3-methylcholanthrene as newborns developed leukemia (albino and C57BL strains), multiple hepatomas (C3H males), and multiple adenomas of the sebaceous glands (albino and C3H mice). Leukemia usually occurred 9 to 16 weeks after injection and was rapidly fatal.—J. Nat. Cancer Inst. 26: 651–679, 1961.

THE INDUCTION of tumors by chemical carcinogens was studied intensively many years ago, particularly in the 1930's and 40's. The literature on this subject has been reviewed (*1, 2*) and compiled in tabular form (*3, 4*).

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[4] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

In these earlier studies, adult animals were generally used, and the age of the animal at the time of administration of carcinogen was not considered critical. A few reports, however, suggested that young animals, *i.e.*, embryos, newborns, sucklings, or young adults, were more susceptible than older animals (*5–11*). On the other hand, some reports indicated that young animals were less responsive to carcinogens than adults (*12–15*). Within the last few years, newborn animals have been widely used as a sensitive test system to assay cell-free extracts of mammalian tissues for ability to induce tumors (*16*). Because of these reports, a re-examination of chemical carcinogenesis was undertaken with the use of newborn animals. This report will present data on the carcinogenic response in noninbred albino and inbred C3H and C57BL mice which, as newborns, were administered a single, subcutaneous, relatively low dose of either dibenz[*a*,*h*]anthracene or 3-methylcholanthrene. A preliminary report of this work has been presented (*17*).

While these studies were in progress, 2 other laboratories reported that suckling and newborn mice were more susceptible than adult mice to tumor induction by chemical carcinogens. Klein (*18*, *19*) obtained an increased incidence of induced liver tumors by initiating treatment (repeated oral administration) with 2-acetylaminofluorene or 3-methylcholanthrene in suckling mice 8 days of age, and Pietra *et al.* (*20*) reported that mice receiving injections as newborns of 9,10-dimethyl-1,2-benzanthracene developed a higher incidence of malignant lymphoma in a shorter time than would be expected in mice receiving injections as adults.

MATERIALS AND METHODS

Purified preparations of dibenz[*a*,*h*]anthracene and 3-methylcholanthrene were used in these experiments.[5] The melting point of dibenz[*a*,*h*]-anthracene was 267.1 to 269.3° C (corrected); the melting point of 3-methylcholanthrene was 179.9–180.6° C (corrected). To prepare for injection, the compounds were ground to a fine powder and dissolved in olive oil with heat (water bath). The concentrations were such that the desired dose was contained in 0.05 or 0.1 ml of olive oil.

The mice used were noninbred albino (General Purpose),[6] and 2 inbred strains, the C57BL/6JN and C3H/P. The mother and her litter were received from the breeding unit within 4 to 16 hours post partum. Animals up to 18 hours old are referred to as newborns in this paper. The mice were weaned at 28 days, sexed, randomized, and housed in groups of approximately 10 to 15 males or females per plastic cage.

Surgical gloves were worn to handle the newborns to minimize the chance of rejection of the litter by the mother and also to protect the hands of the operator from contact with the carcinogen. The entire litter was removed from the cage, the newborns received injections, and the

[5] Kindly supplied to us by Dr. Jonathan Hartwell, National Cancer Institute.
[6] Bred at the National Institutes of Health by systematic randomization of selected stock.

litter returned to the mother. All injections were made with a 27-gauge needle which was inserted under the skin of the back above the tail, and delivery of carcinogen was made into the subcutaneous tissue in the area between the shoulder blades. Any seepage from the injection site was wiped off immediately.

The animals were weighed once a week and inspected for subcutaneous lesions or other abnormalities at that time. Mice with subcutaneous tumors or other abnormalities were kept under close surveillance and, unless they became moribund, they were not killed until the next scheduled sacrifice date. At predetermined intervals after injection of carcinogen, groups of mice were killed with ether and autopsied. Any animal which had a subcutaneous tumor or which appeared sick was chosen, and the remainder of the group were picked at random from the apparently healthy animals. In any one group, 70 to 100 percent were in the latter category.

At autopsy, the lungs were expanded by injection of Zenker-acetic fixative through the trachea. After fixation overnight and washing for 24 hours in running water, the tissues were placed in 70 percent alcohol. At this time, the lungs were inspected for tumor nodules, and the number of surface nodules per lung was recorded and used in the tabulation of data. Several pieces of tissue were taken from each lung and prepared for histologic examination as paraffin sections stained with hematoxylin and eosin. In a few instances, pulmonary tumors not detected on gross inspection were found in microscopic sections, and in these cases the microscopic tumor count was used in tabulation. In treated groups, lung and subcutaneous tumor (or tissue from the site of injection) were removed routinely for microscopic examination. Any other tumors or tissues which appeared abnormal on gross inspection were also examined microscopically, and samples of all tissues were taken at the termination of an experiment. In the untreated controls, lung, liver, spleen, kidney, adrenal gland, pancreas, salivary glands, thymus, gonads, and lymph nodes were taken routinely for histologic study, and intestine, bladder, and other tissues were taken occasionally.

RESULTS

Acute Toxicity of Dibenz[*a*,*h*]anthracene and 3-Methylcholanthrene in Newborn Mice

The levels of carcinogens used in these experiments caused very little acute toxicity. In mice which, as newborns, received a single subcutaneous injection of either 0.06 mg dibenz[*a*,*h*]anthracene or 0.1 mg 3-methylcholanthrene, the mortality (28 days) was less than 10 percent in noninbred albino mice, 10 to 20 percent in C3H/P strain, and 15 to 40 percent in the C57BL/6JN strain. Deaths usually occurred within the first few days of life and were confined to 1 or 2 litters. The mortality appeared to be related not to the toxicity of the drug, but to the competency of the female to care for her young. For example, the litter size was

smaller in the C57BL than in the albino and C3H mice, and the C57BL mothers were more excitable and intolerant to handling of their young. As a result, several of the C57BL females destroyed some of their offspring in transit from the breeding area, and others promptly killed them after injection. There was no difference in mortality rates of control and treated mice, and dose-response experiments failed to demonstrate any relationship between dose level and mortality.

The weight of mice given the carcinogens was roughly 5 to 10 percent less than that of untreated mice throughout the 6-month observation period. The growth of a few treated mice of all strains was stunted (7 to 10 g in 4 weeks) and usually the runts either died or were killed by the mother. Stunted mice which survived remained small for about 8 weeks, but eventually attained a body weight of about 70 to 80 percent of the average of the group At autopsy, the organs of these animals were not appreciably different from those of the others in their group. In a few cases among all 3 strains, in control as well as in carcinogen-treated animals, the mother died of undetermined cause.

Differences were noted in the local reactions to the carcinogens in newborns as compared to suckling and adult mice. Newborns, in contrast to the older groups, did not develop an inflammatory reaction or ulcer formation at the site of injection of dibenz[*a*,*h*]anthracene or 3-methylcholanthrene. Frequently, however, the emergence of hair over the shoulders was delayed or sparse in the mice treated as newborns, and in 10 to 30 percent of these animals the injection site remained nearly free of hair during the entire observation period.

Induction of Pulmonary Tumors in Noninbred Albino Mice Administered Dibenz[*a*,*h*]anthracene or 3-Methylcholanthrene as Newborns

A high incidence of multiple pulmonary tumors was induced in noninbred albino mice given as newborns a single subcutaneous injection either of 0.06 mg dibenz[*a*,*h*]anthracene or of 0.1 mg 3-methylcholanthrene (table 1). Four experiments with each compound were performed over a period of about 18 months and the data for the individual experiments are listed separately in the table to indicate the degree of reproducibility of the effects obtained. The incidence of tumors of the lung and the mean nodule count at intervals after injection of the carcinogens are given in the table. Most of the animals were killed on schedule at 8, 16, and 24 weeks after injection, and only a few mice died or were killed at other times. In such instances (all tables) these mice have been included with animals killed at the next scheduled date.

Since comparable data were obtained with dibenz[*a*,*h*]anthracene and 3-methylcholanthrene, the results will be considered together. As shown in table 1, about 50 percent of the mice developed pulmonary tumors by 8 weeks after injection, 83 percent by 16 weeks, and 96 percent by 24 weeks. The number of tumors per mouse also increased with time

TABLE 1.—Induction of pulmonary tumors in noninbred albino mice given chemical carcinogen as newborns

Experiment number*	0.06 Mg dibenz[a,h]anthracene†			0.1 Mg 3-methylcholanthrene†		
		Pulmonary tumors			Pulmonary tumors	
	Number mice killed	Incidence (percent)	Mean nodule count‡	Number mice killed	Incidence (percent)	Mean nodule count
	8 Weeks after administration of carcinogen					
1	0	—	—	0	—	—
2	5	60	6	5	80	8
3	8	38	2	8	75	9
4	10	40	3	10	60	3
Mean		43	3		70	6
	16 Weeks after administration of carcinogen					
1	3	100	15	4	100	44
2	6	100	20	10	70	28
3	10	70	5	10	100	44
4	14	79	10	13	77	4
Mean		82	11		84	26
	24 Weeks after administration of carcinogen					
1	6	83	27	5	80	20
2	11	100	23	10	100	15
3	11	100	24	8	100	36
4	0	—	—	0	—	—
Mean		96	24		96	23

*Performed at different times between 1/28/58 and 5/13/59.
†Given as a single subcutaneous injection in 0.1 ml olive oil (expts. 1 and 2) or in 0.05 ml olive oil (expts. 3 and 4).
‡Calculated on the basis of total number of mice killed, not just number of mice with pulmonary tumors.

from a mean nodule count of 3 to 6 at 8 weeks to a count of more than 20 by 24 weeks after injection. The data for mice given injections of these carcinogens as newborns did not vary substantially between experiments and indicate good reproducibility both in incidence of pulmonary tumors and in number of tumors per mouse at any given time after injection.

No pulmonary tumors were found in 70 untreated, noninbred, albino control mice killed at 24 weeks of age; 17 of 98 control mice killed at 44 weeks of age had tumors of the lung (mean nodule count 0.2) and 1 had an adenoma of the bile duct.

Dose Response (Pulmonary Tumors) in Noninbred Albino Mice Administered Dibenz[a,h]anthracene or 3-Methylcholanthrene as Newborns

The dose response, as measured by induction of pulmonary tumors, was determined after administration of a single subcutaneous injection of either dibenz[a,h]anthracene or 3-methylcholanthrene to newborn mice. In this study, 40 litters of newborn noninbred albino mice were randomly assigned by litter among 5 logarithmically spaced dose levels of each carcinogen. The results with dibenz[a,h]anthracene are summarized in table 2 and with 3-methylcholanthrene in table 3. Other tumors which were observed (in addition to pulmonary tumors) are in-

TABLE 2.—Dose response (tumor induction) in noninbred albino mice given dibenz[*a*,*h*]anthracene as newborns

Dose* (mg)	Number of mice	Pulmonary tumors: Number with tumors	Pulmonary tumors: Mean nodule count†	Pulmonary tumors: Individual nodule count‡	Other neoplasms	Number with tumor
8 Weeks after administration of carcinogen						
0. 18	8	5	4. 5	0^1, 3^2, 9^2, 12	None	—
0. 06	8	5	2. 0	0^3, 1^3, 2, 11	None	—
0. 02	10	8	2. 0	0^2, 1^2, 2^3, 4^3	None	—
0. 0067	8	2	0. 4	0^6, 1, 2	None	—
0. 0022	8	3	0. 8	0^5, 1^2, 4	None	—
16 Weeks after administration of carcinogen						
0. 18	8	8	39. 0	2, 7, 13, 16, 22, 55, 96, 98	None	—
0. 06	10	7	5. 2	0^3, 1, 3, 4, 7, 9, 13, 15	Fibrosarcoma, s.c.	1
0. 02	10	10	10. 5	1^2, 3^2, 15, 17, 22, 39	None	—
0. 0067	9	2	0. 2	0^7, 1^2	Fibrosarcoma, s.c.	1
0. 0022	8	3	0. 8	0^5, 1^2, 4	None	—
24 Weeks after administration of carcinogen						
0. 18	7	7	40. 5	13, 19, 36, 57, 78, 80, 122	Fibrosarcoma, s.c.	2
0. 06	11	11	22. 7	1, 3, 6, 12, 14, 20^2, 21, 40, 90	None	—
0. 02	11	9	8. 4	0^2, 1^2, 2, 3^2, 19^2, 41	Fibrosarcoma, s.c.	1
0. 0067	9	5	1. 0	0^4, 1^4, 5	None	—
0. 0022	12	4	0. 4	0^8, 1^3, 2	None	—

*Given as a single subcutaneous injection in 0.05 ml olive oil.

†Calculated on basis of total number of mice killed, and not just number of mice with pulmonary tumors.

‡Exponent indicates number of animals (where more than one) having a particular nodule count.

TABLE 3.—Dose response (tumor induction) in noninbred albino mice given 3-methylcholanthrene as newborns

Dose* (mg)	Number of mice	Pulmonary tumors: Number with tumors	Pulmonary tumors: Mean nodule count†	Pulmonary tumors: Individual nodule count‡	Other neoplasms	Number with tumor
8 Weeks after administration of carcinogen						
0.3	8	8	18.7	3, 7, 9, 14^2, 27, 30, 46	None	—
0.1	8	6	9.4	0^2, 3, 4, 5^2, 9, 49	None	—
0.033	9	4	5.9	0^5, 3^2, 12, 35	None	—
0.011	9	2	0.4	0^8, 1, 2	None	—
0.0037	0	—	—		—	—
16 Weeks after administration of carcinogen						
0.3	10	10	78.1	14, 15, 47, 64, 66, 69, 86, 87, 131, 202	Fibrosarcoma, s.c.	3
					Leukemia	2
0.1	10	10	43.5	4, 6, 7, 11, 18, 26, 35, 78, 88, 162	Fibrosarcoma, s.c.	1
					Leukemia	1
0.033	8	8	35.2	9, 11, 14, 16, 28, 49, 50, 104	Fibrosarcoma, s.c.	3
0.011	11	8	1.8	0^3, 1^4, 2, 3, 4, 7	Fibrosarcoma, s.c.	1
0.0037	8	0	—	0^8	None	—
24 Weeks after administration of carcinogen						
0.3	8	8	102.4	32, 48, 76, 87, 99, 140, 168, 169	Fibrosarcoma, s.c.	1
					Adenoma, sebaceous glands	1
					Leukemia	1
0.1	8	8	34.2	15, 21, 25, 30, 40, 43, 49, 61	None	—
0.033	9	9	29.0	1, 10, 17, 19, 25, 32, 48, 49, 60	Fibrosarcoma, s.c.	3
0.011	11	11	10.4	2, 4, 7, 8^2, 9^2, 13, 14, 16, 24	Fibrosarcoma, s.c.	2
0.0037	6	0	—	0^6	None	—

*Given as a single subcutaneous injection in 0.05 ml olive oil.
†Calculated on basis of total number of mice killed, and not just number of mice with pulmonary tumors.
‡Exponent indicates number of animals (where more than one) having a particular nodule count.

cluded in the tables, but consideration of these lesions is deferred to a later section of this report.

The percentage of mice developing pulmonary tumors and the number of tumor nodules per mouse increased with dose and with time after injection. As shown in table 2, the lowest dose of dibenz[*a*,*h*]anthracene (0.0022 mg) induced pulmonary tumors in 38 percent of the mice by 8 weeks, with a mean nodule count of 0.8. At this dose, neither the incidence nor the mean nodule count increased with time over the 24-week observation period. Maximum response [7] was obtained by dose levels of 0.02, 0.06, and 0.18 mg dibenz[*a*,*h*]anthracene. The mean nodule count, however, was considerably higher at all time intervals with the 0.18 mg dose, though the incidence of tumors of the lung was about the same for all 3 doses and showed no increase with time. By 24 weeks, when pulmonary tumors had developed in virtually all the animals which received 0.02 mg or more of dibenz[*a*,*h*]anthracene, the mean nodule count still varied with dose, ranging from 8 at the lowest dose to 40 at the highest. The mean nodule count, therefore, appears to be a more sensitive index of intensity of carcinogenic response than tumor incidence.

The dose response to 3-methylcholanthrene (table 3) was similar to that with dibenz[*a*,*h*]anthracene. Again the incidence of pulmonary tumors and the number of tumors per mouse increased, within limits, with dose and with time. No pulmonary tumors were found in mice given the lowest dose (0.0037 mg), but maximum response (100% incidence of pulmonary tumors in 24 weeks) was obtained at all higher dose levels (0.011 mg to 0.3 mg). The highest dose, 0.3 mg 3-methylcholanthrene, induced pulmonary tumors in all the mice within 8 weeks with a mean nodule count of 18.7 at that time. By 16 weeks, the nodule count had increased to 78, and by 24 weeks to 102 nodules per mouse, which suggested a sustained carcinogenic effect. With 3-methylcholanthrene as with dibenz[*a*,*h*]anthracene, the mean nodule count appears to be a more sensitive measure of dose response than the incidence of mice with pulmonary tumors.

Effect of Age at Injection on Pulmonary-Tumor Injection

A direct comparison was made of the latent period, incidence, and mean nodule count of pulmonary tumors induced in mice of different ages at the time of administration of either 0.06 mg dibenz[*a*,*h*]anthracene or 0.1 mg 3-methylcholanthrene. In this study, 24 litters of newborn noninbred albino mice were allocated at random to treatment groups which were given a single subcutaneous injection of carcinogen either as newborns, as sucklings (1 or 3 weeks of age), or as young adults (6 weeks of age). The mice were killed at 8, 16, and 24 weeks after injection and examined for pulmonary tumors and other pathologic lesions. The results are summarized in table 4.

[7] To facilitate comparison, maximum response has been arbitrarily defined as 90 to 100 percent incidence of lung tumors by 24 weeks after administration of carcinogen.

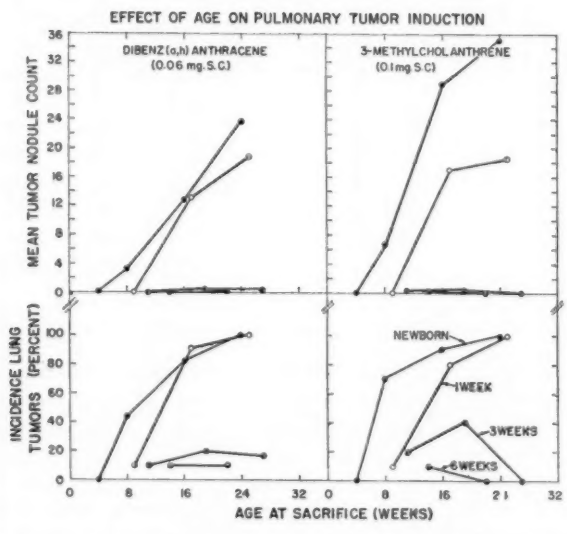
TABLE 4.—Effect of age on susceptibility of noninbred albino mice to tumor induction by chemical carcinogens

Age at injection (weeks)	0.06 Mg dibenz[a,h]anthracene					0.1 Mg 3-methylcholanthrene*				
	Pulmonary tumors				Fibrosarcoma†	Pulmonary tumors				Fibrosarcoma†
	Number of mice	Number with tumor	Mean nodule count‡	Individual nodule count§	Number with tumor	Number of mice	Number with tumor	Mean nodule count	Individua nodule count	Number with tumor
8 Weeks after administration of carcinogen										
Newborn	10	4	3.1	0^6, 1^2, 8, 21	0	10	6	2.2	0^4, 1, 2^2, 4, 6, 7	0
1	10	1	0.1	0^9, 1	0	10	1	0.1	0^9, 1	0
3	10	1	0.1	0^9, 1	0	10	2	0.3	0^8, 1, 2	0
6	10	1	0.1	0^9, 1	0	10	1	0.3	0^9, 3	0
16 Weeks after administration of carcinogen										
Newborn	14	11	10.3	0^3, 1, 3, 7^2, 13, 14, 16^2, 17, 19, 29	0	13	10	4.2	0^3, 1^2, 2, 4^2, 8, 10, 12, 13	2
1	11	10	13.2	0, 2, 4^2, 10, 11, 14, 16, 18, 23, 43	2	10	8	17.1	0^2, 2, 4, 9^2, 30, 33, 37, 47	2
3	10	2	0.4	0^8, 1, 3	1	12	5	0.4	0^7, 1^5	2
6	10	1	0.2	0^9, 2	1	14	0	0	0^{14}	4
24 Weeks after administration of carcinogen										
1	4	4	18.8	2, 10, 16, 45	1	8	8	18.6	2, 7^2, 8, 20, 23, 32, 50	0
3	6	1	0.2	0^5, 1	1	4	0	0	0^4	1

*Given as a single subcutaneous injection in 0.05 ml olive oil.
†At site of subcutaneous injection. In this experiment, no other neoplasms were observed.
‡Calculated on basis of total number of mice killed, not just number of mice with pulmonary tumors.
§Exponent indicates number of animals (where more than one) having a particular nodule count.

Within 8 weeks after injection, pulmonary tumors had developed in 40 percent of the mice given dibenz[*a*,*h*]anthracene as newborns, and in only 10 percent of those given the carcinogen at 1, 3, or 6 weeks of age. The mean nodule count of the different age groups at this time was 3.1 for the newborns and 0.1 for the 1-, 3-, and 6-week-old groups. By 16 weeks after injection, however, mice given dibenz[*a*,*h*]anthracene at 1 week of age had a somewhat higher incidence (91 vs. 78%) and mean nodule count (13.2 vs. 10.3) than animals given the carcinogen as newborns. In contrast, the mice treated at 3 and 6 weeks of age showed little or no increase in either incidence or mean tumor count throughout the observation period. As shown in the table, comparable results were obtained with 3-methylcholanthrene.

In text-figure 1, the percent incidence of pulmonary tumors and the mean nodule count of mice administered dibenz[*a*,*h*]anthracene or 3-methylcholanthrene as newborns or at 1, 3, or 6 weeks of age are plotted against chronological age of the mice when they were killed rather than time after injection of carcinogen. In these graphs, the total experience with newborns was used instead of data from the single experiment shown in table 4. The text-figure illustrates the similarities as well as differences among age groups. In most respects, the pattern and intensity of response of the newborn and the 1-week-old groups were comparable to



TEXT-FIGURE 1.—Induction of pulmonary tumors with chemical carcinogens. Effect of age on latent period, incidence, and mean number of tumor nodules per mouse. Noninbred albino mice.

●—● Receiving injections as newborns
○—○ Receiving injections at 1 week of age
◐—◐ Receiving injections at 3 weeks of age
◒—◒ Receiving injections at 6 weeks of age

each other, and differed considerably from that of the older age groups. The newborn and 1-week-old mice showed a progressive increase in response to both carcinogens during the subsequent 24-week observation period, particularly in the number of tumor nodules per mouse. The latent period, however, was longer in the 1-week-old animals than in the newborns (16 weeks vs. 8 weeks). These results are in contrast to those obtained with animals given the carcinogen at 3 or 6 weeks of age, in which the response curves remain low and essentially flat throughout the observation period.

The text-figure also illustrates the similarity in response elicited by dibenz[*a*,*h*]anthracene and 3-methylcholanthrene. After a single subcutaneous injection of either carcinogen in newborn mice, the incidence of pulmonary tumors increased sharply to 80 percent or more by 16 weeks, and to about 95 percent by 24 weeks. The number of tumor nodules per mouse also increased rapidly between 8 and 16 weeks, and was still increasing at 24 weeks after injection of either carcinogen. The mean nodule count, however, was consistently higher in animals given 3-methylcholanthrene as newborns than in those given dibenz[*a*,*h*]-anthracene.

Although assays of carcinogenicity in adult animals have been made almost exclusively on a total-dose basis, it is obvious that the order of magnitude of the difference in dose on an mg/kg basis is much greater between newborn and adult mice than between adult animals of different weight and age. This could have a considerable influence on the results, particularly since, both in adults and in newborns, the intensity of the response varies directly with dose. Some of the data contained in tables 2 to 4 have been recalculated, therefore, to permit comparison of responses of different age groups on an mg/kg basis. As shown in table 5, the response in newborns is greater than that in older animals, whether dose is expressed as mg/kg or mg/animal.

Induction of Pulmonary Tumors in C3H/P and C57BL/6JN Mice Administered Dibenz[*a*,*h*]anthracene or 3-Methylcholanthrene as Newborns

Newborn C3H/P and C57BL/6JN mice were given a single subcutaneous injection of either 0.06 mg dibenz[*a*,*h*]anthracene or 0.1 mg 3-methylcholanthrene. Twelve litters of each strain were used, 6 with dibenz-[*a*,*h*]anthracene and 6 with 3-methylcholanthrene. These strains were selected because adult C3H and C57BL mice have been reported to be relatively refractory to lung-tumor induction by chemical carcinogens, even on intravenous administration (*21*, *22*).

The results obtained with newborn C3H/P mice are summarized in table 6. The incidence of pulmonary tumors induced by dibenz[*a*,*h*]-anthracene was 12 percent by 16 weeks after injection, and increased to 62 percent by 24 weeks. During this period, the mean nodule count increased from 0.5 to 3.8 tumors per mouse. With 3-methylcholanthrene,

TABLE 5.—Effect of age on the weight-adjusted susceptibility of noninbred albino mice to pulmonary tumor induction*

Age at injection (weeks)	Dose		Pulmonary tumors					
			8 weeks		16 weeks		24 weeks	
	mg/animal	mg/kg	Incidence (%)	Mean† nodule count	Incidence (%)	Mean nodule count	Incidence (%)	Mean nodule count
	Dibenz[*a*,*h*]anthracene, s.c.							
Newborn	0. 02	13. 5	80	2. 0	100	10. 5	82	8. 4
Newborn	0. 0067	4. 5	25	0. 4	22	0. 2	56	1. 0
Newborn	0. 0022	1. 5	38	0. 8	38	0. 8	33	0. 4
1	0. 06	14. 6	10	0. 1	91	13. 2	100	18. 8
3	0. 06	5. 4	10	0. 1	20	0. 4	17	0. 2
6	0. 06	2. 8	10	0. 1	10	0. 2	—	—
	3-Methylcholanthrene, s.c.							
Newborn	0. 033	22. 0	44	5. 9	100	38. 9	100	34. 7
Newborn	0. 011	7. 3	22	0. 4	73	1. 8	100	11. 0
Newborn	0. 0037	2. 5	0	0	0	0	0	0
1	0. 1	25. 6	10	0. 1	80	17. 1	100	18. 6
3	0. 1	9. 3	20	0. 3	42	0. 4	0	0
6	0. 1	4. 8	10	0. 3	0	0	—	—

*Data taken from tables 4–7.

†Calculated on basis of total number of mice killed, not just number of mice with pulmonary tumors.

TABLE 6.—Induction of tumors in C3H/P mice given dibenz[*a*,*h*]anthracene or 3-methylcholanthrene as newborns

Weeks after injection	Number of mice	Pulmonary tumors: Number with tumor	Pulmonary tumors: Mean nodule count*	Pulmonary tumors: Individual nodule count†	Other neoplasms	Number with tumor
			0.06 Mg dibenz[*a*,*h*]anthracene‡			
16	16	2	0. 5	0^{14}, 3, 5	Fibrosarcoma, s.c.	3
24	21	13	3. 8	0^{8}, 1^{4}, 3^{3}, 4, 7^{2}, 9, 14, 22	Fibrosarcoma, s.c.	9
32	12	8	3. 1	0^{4}, 1^{4}, 2^{2}, 3, 9, 18	Fibrosarcoma, s.c.	1
			0.1 Mg 3-methylcholanthrene‡			
16	14	6	1. 8	0^{8}, 1^{2}, 2, 5, 7, 9	Fibrosarcoma, s.c. Adenoma, sebaceous glands	9 1
24	16	11	13. 4	0^{5}, 1^{4}, 2^{2}, 6, 13, 14, 80, 92	Fibrosarcoma, s.c. Adenoma, sebaceous glands Hepatoma§	6 2 1
32	10	8	11. 1	0^{2}, 2, 3, 7^{2}, 16^{2}, 25, 35	Fibrosarcoma, s.c. Adenoma, sebaceous glands Hepatoma§	2 3 5

*Calculated on basis of total number of mice killed, not just number of mice with pulmonary tumors.
†Exponent indicates number of animals (where more than one) having a particular nodule count.
‡Given as a single subcutaneous injection in 0.05 ml olive oil.
§Hepatomas occurred in male mice only.

43 percent of the animals had developed pulmonary tumors by 16 weeks (mean nodule count, 1.8), 67 percent by 24 weeks (mean nodule count, 13.4), and 80 percent by 32 weeks (mean nodule count, 11.1).

Four of 128 control C3H/P mice autopsied at 30 to 32 weeks of age had a single pulmonary tumor nodule, 1 had 4 tumors of the lung, and 1 mouse had a teratoma of the ovary. Of 37 mice autopsied at 42 weeks of age, 2 had a single pulmonary tumor, and 1 of 17 males had a single hepatoma.

The data for newborn C57BL/6JN mice are summarized in table 7. Pulmonary tumors were found in 2 of 11 mice killed 24 weeks after dibenz[*a*,*h*]anthracene, and in 5 of 11 animals killed 32 weeks after 3-methylcholanthrene. The mean number of tumors per mouse was 0.2 and 0.7, respectively. The incidence of spontaneous tumors is low in the C57BL/6JN strain. No tumors were found in mice autopsied at 24 weeks of age. Of 159 mice autopsied at 2 years of age, 1 had a single tumor of the lung, 1 a hepatoma, another a Hodgkin-like lesion, and 2 reticulum-cell sarcomas.

TABLE 7.—Induction of tumors in C57BL/6JN mice given dibenz[*a*,*h*]anthracene or 3-methylcholanthrene as newborns

Weeks after injection	Number of mice	Pulmonary tumors: Number with tumor	Pulmonary tumors: Mean nodule count*	Pulmonary tumors: Individual nodule count†	Other neoplasms	Number with tumor
0.06 Mg dibenz[*a*,*h*]anthracene‡						
16	10	0	—	—	Fibrosarcoma, s.c.	2
24	11	2	0.2	0^9, 1^2	Fibrosarcoma, s.c.	1
0.1 Mg 3-methylcholanthrene‡						
16	13	1	0.1	0^{12}, 1	Fibrosarcoma, s.c.	4
24	3	1	0.3	0^2, 1	Fibrosarcoma, s.c.	2
					Leukemia	1
32	11	5	0.7	0^6, 1^3, 2, 3	Fibrosarcoma, s.c.	2

*Calculated on basis of total number of mice killed, not just number of mice with pulmonary tumors.
†Exponent indicates number of animals (where more than one) having a particular nodule count.
‡Given as a single subcutaneous injection in 0.05 ml olive oil.

Induction of Nonpulmonary Tumors in Mice Administered Dibenz-[*a*,*h*]anthracene or 3-Methylcholanthrene as Newborns

The experiments reported in this paper were designed primarily to furnish information on the induction of pulmonary tumors in mice given a single subcutaneous injection of dibenz[*a*,*h*]anthracene or 3-methylcholanthrene as newborns. Data on the incidence of other lesions were obtained incidentally and are not quantitative. The total experience (including data presented in previous tables) on induction of all types of tumors in noninbred albino, C3H/P, and C57BL/6JN mice receiving injections as newborns is summarized in table 8. Only lesions which were definitely tumors and occurred in more than 1 animal are included in the table.

TABLE 8.—Tumors induced in mice given dibenz[*a*,*h*]anthracene or 3-methylcholanthrene as newborns (Summary)

Strain	Dose (mg/mouse) s.c.	Total number of mice*	Number of mice with tumor				
			Adenoma, lung	Fibrosarcoma, s.c.	Leukemia	Hepatoma	Adenoma, sebaceous glands
Dibenz[*a*,*h*]anthracene							
Noninbred albino	0. 18	23	20	2	0	0	0
" "	0. 06	93	66	18	1	0	0
" "	0. 02	31	27	0	0	0	0
" "	0. 0067	26	9	2	0	0	0
" "	0. 0022	28	10	0	0	0	0
" "	*Total*	*201*	*132*	*22*	*1*	*0*	*0*
C3H/P	0. 06	49	23	13	0	0	0
C57BL/6JN	0. 06	22	2	3	0	0	0
3-Methylcholanthrene							
Noninbred albino	0. 3	26	26	4	3	0	1
" "	0. 1	91	66	8	2	0	0
" "	0. 033	26	21	6	0	0	0
" "	0. 011	30	21	3	0	0	0
" "	0. 0037	14	0	0	0	0	0
" "	*Total*	*191*	*134*	*21*	*5*	*0*	*1*
C3H/P	0. 1	40	25	17	0	6	6
C57BL/6JN	0. 1	27	7	8	1	0	0

*Age of mice when killed: noninbred albino, 8 to 24 weeks; C57BL/6JN, 16 to 24 weeks; C3H/P, 16 to 32 weeks.

3-Methylcholanthrene induced a wider variety of neoplasms than dibenz[*a*,*h*]anthracene. Pulmonary tumors, subcutaneous fibrosarcomas, and leukemia were produced by both carcinogens, while hepatomas and adenomas of the sebaceous glands were induced only by 3-methylcholanthrene. The fibrosarcomas occurred as single tumors and arose on the dorsal surface of the mouse, apparently at or near the site of injection.

Strain differences in susceptibility of specific tissues to the carcinogenic action of dibenz[*a*,*h*]anthracene and 3-methylcholanthrene were apparent. Pulmonary tumors and leukemia were obtained more frequently in albino mice than in C3H or C57BL animals, and hepatomas were induced only in C3H males. Subcutaneous fibrosarcomas were found more frequently, perhaps, in the C3H and C57BL strains than in albino mice,[8] though the latent period was about the same in all 3 strains (earliest fibrosarcoma noted in 9 weeks).

Gross and Microscopic Description of Tumors Induced in Mice Administered Dibenz[*a*,*h*]anthracene or 3-Methylcholanthrene

Pulmonary tumors.—The pulmonary tumors induced in mice given dibenz[*a*,*h*]anthracene or 3-methylcholanthrene as newborns were similar to those induced in older mice and to spontaneous tumors of the lung (*24–26*). The typical gross and microscopic appearance of the pulmonary tumors is shown in figures 1, 2, and 3. They were circumscribed, nonencapsulated neoplasms with an adenomatous pattern and arose from proliferation of the alveolar septal cells. In the early stages of development of a tumor nodule, these cells formed loose aggregates which retained in part the structure of alveoli. In the fully developed tumor nodule, the normal alveolar structure of the lung was obliterated, and the cells formed a compact mass which compressed but did not invade adjacent normal lung. Serial sections were made of some of the lungs to study the development of tumor nodules. Usually a single lung contained tumors in all stages of development which suggested a continuing process of tumor formation after a single exposure to carcinogen.

Subcutaneous fibrosarcomas.—Grossly and microscopically, the fibrosarcomas induced in newborn mice by dibenz[*a*,*h*]anthracene and 3-methylcholanthrene were similar to those induced in adult animals (*27–29*). The tumors were usually well differentiated but often invaded adjacent tissues such as skin, muscle, peritoneum, rib cage, salivary glands, eyelids, and lymph nodes. Frequently, hyperchromatic giant cells, multinucleated straplike cells, and other bizarre myogenous elements were contained within the tumor. Transition could be traced from these forms to more differentiated cells and finally to adjacent bundles of intact muscle. These abnormal elements were obviously not part of the tumor,

[8] Life table analyses (*25*) of the data on subcutaneous fibrosarcomas in mice given dibenz[*a*,*h*]anthracene or 3-methylcholanthrene as newborns suggest the following incidence: for noninbred albino mice, 5 to 20 percent in 24 weeks; for C57BL/6JN mice, about 38 percent in 32 weeks; and for C3H/P mice, roughly 34 percent in 32 weeks. In noninbred albino mice treated with carcinogens at 1 or 3 weeks of age, the suggested incidence is 12 to 35 percent in 24 weeks; in noninbred albino mice treated at 6 weeks of age, the incidence is 10 to 30 percent in 16 weeks.

but represented fragments of regenerating striated muscle. In some instances, fibrosarcomas appeared to originate in the perimysium of the panniculus carnosus (figs. 4, 5, and 6), but in others they arose adjacent to small cystic areas in the subcutaneous tissue. In the latter, the tumors apparently arose from fibrocytes of the adipose tissue and not from fat cells.

Leukemia.—The leukemia induced in mice which received an injection of dibenz[*a*,*h*]anthracene or 3-methylcholanthrene as newborns resembled the lymphocytic leukemia obtained in adult mice after irradiation (*30*), skin painting (*31*), or oral administration (*32*) of carcinogens. The thymus was always enlarged and showed lymphosarcomatous transformation. As a rule, there was also leukemic infiltration of the kidney, lung, and liver.

Hepatomas.—The hepatomas appeared grossly as multiple, small, pale nodules and were similar in appearance to spontaneous hepatomas previously described (*33*). The typical tumor cell was small and showed increased cytoplasmic basophilia with frequent clear areas in the cytoplasm.

Adenomas of the sebaceous glands.—The sebaceous-gland adenomas on the surface of the skin were papillary excrescences, generally multiple, measuring 1 to 5 mm in diameter (fig. 7). The tumor nodule consisted of a closely packed group of sebaceous glands supported by a small amount of collagenous stroma (fig. 8). The sebaceous adenoma was attached by a short stalk through which the stromal collagen was continuous with the dermal collagen of the skin. The adenoma was covered by a thickened epidermis which was usually 4 to 15 cells deep. Cystic spaces lined by squamous epithelium, presumably distended hair follicles, were often present in these tumors, and frequently groups of hypertrophied hair follicles could be seen in the adjacent skin. Mitotic figures were not seen. Sections stained with Oil Red O showed abundant fat within the sebaceous cells.

DISCUSSION

It is generally agreed that the susceptibility of an inbred strain of mice to the induction of a particular tumor reflects the frequency of spontaneous occurrence of the tumor within the strain (*34–37*). The frequency of spontaneous tumors of the lung has been rated as moderately high (40 to 50%) in Swiss, medium (5 to 10%) in C3H, and low (<1%) in C57BL/6JN mice (*38*, *39*). The experiments reported here have established the susceptibility to lung-tumor induction of newborn noninbred albino, C3H/P, and C57BL mice in the order listed. Although the older literature contains many references to induction of pulmonary tumors by dibenz[*a*,*h*]anthracene and 3-methylcholanthrene in adult mice of the C3H and C57BL strains, differences in observation time, dosage, vehicle, and route of administration preclude quantitative com-

parison with the results obtained in newborns. However, the response obtained in newborn C3H and C57BL mice was greater after subcutaneous administration of dibenz[*a*,*h*]anthracene or 3-methylcholanthrene than that reported in the literature in adult mice of these strains given 5 to 10 times more carcinogen by intravenous route (*21*, *22*). More precise information on the influence of age on induction of pulmonary tumors was obtained by a direct comparison of the responses of newborn, suckling, and young adult albino mice to dibenz[*a*,*h*]anthracene or 3-methylcholanthrene. The latent period was shorter, the incidence was greater, and the mean nodule count was higher in newborn than in older mice. This was true whether dose was expressed on an mg/kg basis or as mg/animal.

The pulmonary tumors arose from the alveolar septal cells which receive their blood supply from the pulmonary system. Since the blood flow in the pulmonary system is equal to the flow in the entire systemic circulation, and since the bronchial arteries receive only a small portion of the systemic blood flow, it follows that the septal cells probably have a greater exposure to a circulating carcinogen than the cells of the bronchial mucosa. However, other factors, such as the intrinsic susceptibility of different cell types to neoplastic change, undoubtedly play an important part in determining which cell type will become malignant. A continuing process of pulmonary tumor formation was apparent from study of the histologic sections (including serial sections) and from gross observations. All the tumors did not arise at the same time. Instead, there was a progressive increase in size and number of tumor nodules with time, and even at 24 weeks after injection of carcinogen there were numerous proliferating septal cells which had not yet reached the tumor stage. Apparently there was some alteration in the alveolar septal cells or in the host animal which, over a period of time, produced a progression of neoplastic changes in the lung.

Collection of data on subcutaneous fibrosarcomas and other tumors was ancillary to that on pulmonary tumors and does not allow quantitative comparisons. However, subcutaneous fibrosarcomas as well as pulmonary tumors were produced in all strains of mice by both compounds. The presence in the same animal of a subcutaneous fibrosarcoma and of pulmonary tumors appeared to occur at random, and no consistent relation between the two neoplasms could be established. Two sites of origin of subcutaneous fibrosarcomas have been identified: the perimysium of the panniculus carnosus, and small cysts in the subcutaneous adipose tissue where the tumors appear to arise from fibroblasts in the cyst wall. Other sites, however, cannot be ruled out, since many of the tumors were too large at the time of autopsy to determine their origin. A number of classifications has been proposed for subcutaneous tumors induced by carcinogenic hydrocarbons, based on the presence of smooth muscle elements, rhabdomyoblasts, and pleomorphic cells (*40*, *27*). In the present study, giant or pleomorphic cells and multinucleated, straplike cells could usually be traced directly to remnants of invaded striated muscle, and the malignant cells appeared to arise from fibrocytes. The

simple classification of this tumor as fibrosarcoma is in agreement with the classification proposed by Lewis (*28*), and does not exclude the possibility that the myoblasts may dedifferentiate into malignant spindle cells indistinguishable from fibrosarcoma cells. In any case, the fibrosarcomas arising in the perimysium and subcutaneous tissue were similar except for the early invasion of muscle by tumors arising in the perimysium.

Spontaneous or induced adenomas of the sebaceous glands are uncommon in mice. Klein (*19*) obtained sebaceous-gland adenomas in 3 of 55 inbred albino mice given 15 mg of 3-methylcholanthrene (repeated oral administration) beginning at 8 days of age, but none in 110 adult mice given 5 times as much of the carcinogen. In our experiments, 1 noninbred albino and 6 C3H/P mice administered 0.1 mg of 3-methylcholanthrene subcutaneously as newborns developed sebaceous-gland adenomas within 24 weeks. It is possible that, in these mice as in all the newborns, there may have been some seepage of carcinogen at the site of subcutaneous injection. In the majority of animals, however, the sebaceous-gland adenomas were located on the ventral and lateral surfaces as well as on the dorsal surface where the injection was made.

Leukemia was observed only in mice which were given the carcinogens as newborns, and was not found in mice 1, 3, or 6 weeks of age at the time of treatment, or in untreated control mice of comparable age. So far as we are aware, induction of leukemia by parenteral administration of dibenz[*a*,*h*]anthracene or 3-methylcholanthrene has not been reported previously, though it has been induced in adult mice by repeated percutaneous (*41*) or oral administration (*32*) of much higher dose levels of 3-methylcholanthrene.

Until recently, liver has proved refractory to tumor induction by polycyclic hydrocarbons. Andervont (*42*) observed hepatomas in C3H mice under 13 months of age after subcutaneous administration of 3-methylcholanthrene, but did not attribute the neoplasms to the carcinogen because of the high incidence of spontaneous hepatomas in old C3H mice (*43*, *44*). Within the past year, Klein (*19*) reported induction of hepatomas in 10 to 12 months in inbred albino mice given 3-methylcholanthrene by oral route. When treatment was initiated in 8-day-old mice (total dose, 15 mg), a higher incidence of hepatomas and a shorter latent period were obtained than when administration of carcinogen was begun in 90-day-old animals (total dose, 60 mg). In our experiments, hepatomas were found in 6 of 11 male C3H mice killed between 24 and 32 weeks after a single subcutaneous injection of 0.1 mg of methylcholanthrene as newborns. No hepatomas were found in control animals killed at comparable age, and these neoplasms are believed to have been induced.

The greater susceptibility to chemical carcinogenesis of newborn mice as compared with older animals may be due to many factors. For example, many enzyme systems in the liver of embryonic, newborn, or young animals have been shown to be qualitatively or quantitatively different from those in adults (*45–48*). Such variation could result in differences in toxicity, rate of degradation, or intermediate metabolism and also, pos-

sibly, in the neoplastic response of the animal to the carcinogen. Furthermore, several investigators have reported a relationship between protein-binding and tumor production by several chemical classes of carcinogens (*49–52*). Differences in amount of carcinogen bound by protein in newborn and adult mice might lead to a higher effective dose in the newborn animal. The observation that newborn mice do not develop the local inflammatory reaction seen in suckling and adult mice after subcutaneous administration of dibenz[*a*,*h*]anthracene or 3-methylcholanthrene may indicate decreased protein-binding in the newborn animals, or increased rate of absorption from the local site.

The minimum dose of dibenz[*a*,*h*]anthracene or 3-methylcholanthrene which will induce tumors in mice has not been determined. Tumors were induced within 24 weeks with 2.2 μg of dibenz[*a*,*h*]anthracene or 11 μg of methylcholanthrene. Experiments to find the minimum effective dose are in progress and will be reported at a later date. Good reproducibility in pulmonary-tumor induction was obtained in mice given a single subcutaneous injection of dibenz[*a*,*h*]anthracene or 3-methylcholanthrene as newborns. This, as well as the enhanced response and the shortened latent period, suggests that use of newborn animals offers a more sensitive system in which to assay material of suspected carcinogenic activity or of low potency. Preliminary work in this laboratory has demonstrated that compounds may be administered without difficulty to newborn mice, rats, and hamsters by oral and intraperitoneal routes, if the volume administered is 0.02 ml or less. The applicability of such a system for other classes of chemical carcinogens is indicated by the published results with urethan (*9*), and 9,10-dimethyl-1,2-benzanthracene (*20*), and is suggested by the results with 2-acetylaminofluorene in sucklings (*18*).

Finally, the implications of the present study as related to man should be considered. This is done with full cognizance of all the errors inherent in extrapolation of data from mouse to man, from a small sample to a larger population, or from one compound to another, even a closely related analogue. In the fields of public health safety and environmental cancer, an age differential in suceptibility to carcinogenic agents would be of utmost importance. Currently, considerable attention has been focused on an apparent age difference in leukemogenic response in humans to ionizing radiation, particularly as regards exposure during fetal life. The possibility of a similar situation in regard to other carcinogenic agents in the human environment should be examined. It would seem of practical value to determine whether a "safe" level of exposure for one age group is a "safe" level for all ages.

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PLATE 85

FIGURE 1.—Gross appearance of lungs of Swiss mouse 16 weeks old given 0.1 mg 3-methylcholanthrene subcutaneously as newborn. × 2

FIGURE 2.—Gross appearance of lungs of 2 Swiss mice 24 weeks old given 0.3 mg 3-methylcholanthrene subcutaneously as newborns. × 2

FIGURE 3.—Microscopic appearance of one of lungs shown in figure 2. Larger tumor nodules are circumscribed and show internal, cordlike arrangement of tumor cells. Small nodules still show, at their margins, partial retention of alveolar structure of lung. Serial sections of lung confirm presence of tumor nodules in all stages of development. Hematoxylin and eosin. × 54

1

2

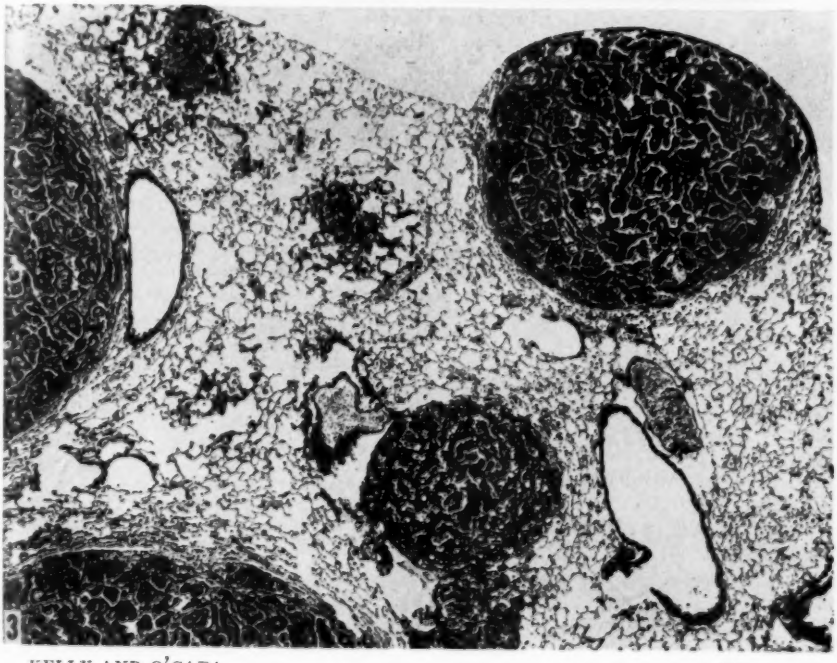
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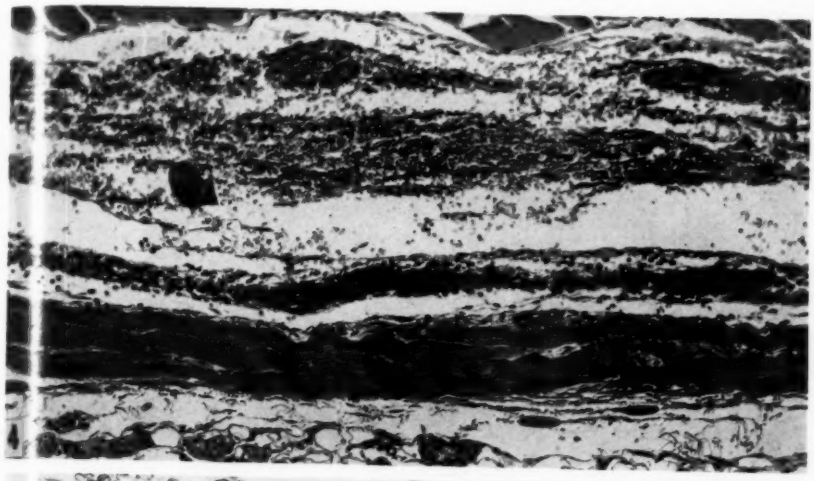
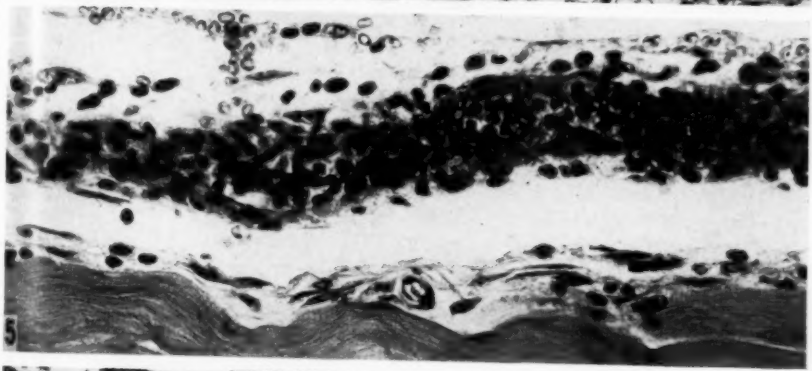
PLATE 86

FIGURE 4.—Premalignant lesion in subcutaneous tissue of a noninbred albino mouse 16 weeks old, given 0.06 mg dibenz[*a*,*h*]anthracene subcutaneously at 1 week of age. This lesion is located in perimysium of panniculus carnosus and adjacent subcutaneous tissue, and extends in linear manner along margin of muscle bundle. Zone of extravasated blood separates the lesion from subcutaneous layer of muscle (*upper* margin of photomicrograph). Hematoxylin and eosin. × 125

FIGURE 5.—Higher magnification of lesion shown in figure 4. Band of fibrous tissue in close apposition to panniculus carnosus shows proliferation and rounding-up of fibrocytes. Hematoxylin and eosin. × 430

FIGURE 6.—Early fibrosarcoma in a C57BL mouse 16 weeks old, given 0.06 mg dibenz[*a*,*h*]anthracene subcutaneously as a newborn. This tumor apparently arose from perimysium of panniculus carnosus. *Note* close interrelationship of tumor and perimysium at *right* of photomicrograph. Numerous hyperchromatic giant cells can be seen within tumor and probably represent damaged or regenerating muscle cells. Many giant cells contain 2 or more nuclei. Hematoxylin and eosin. × 50





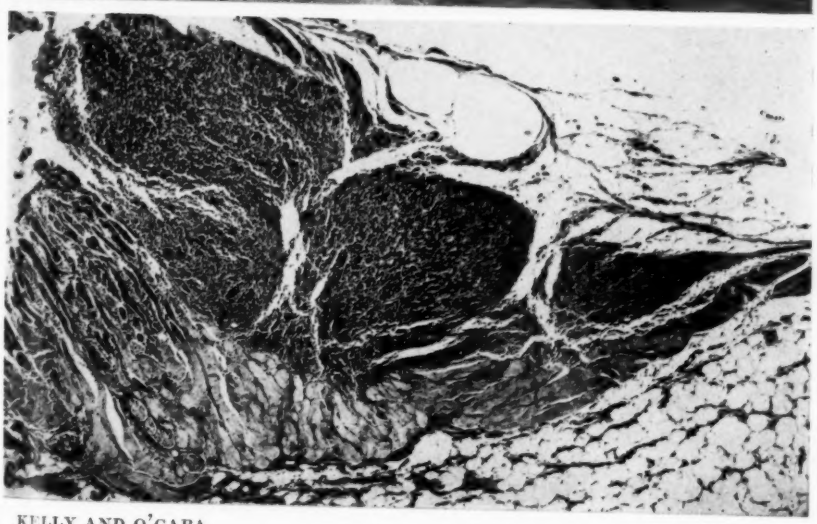
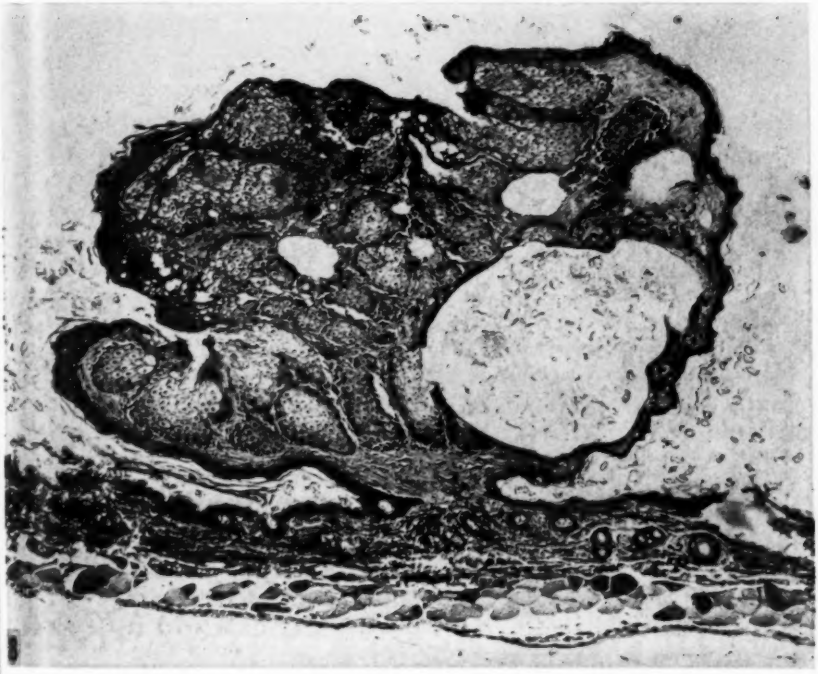


PLATE 87

FIGURE 7.—Multiple sebaceous-gland adenomas in skin of a noninbred albino mouse 24 weeks old, given 0.3 mg 3-methylcholanthrene subcutaneously as a newborn. × 1

FIGURE 8.—Section through 1 of nodules shown in figure 7. Nodule is composed of many closely packed sebaceous glands. It is covered by stratified squamous epithelium and is attached to the skin by a short stalk. Hematoxylin and eosin. × 100



Cytotoxic Action *In Vitro* of Human Serum on the Metabolism of Mouse Ascites Tumor Cells and Counteraction by Endotoxic and Other Polysaccharides [1]

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SUMMARY

The cytotoxic action of human serum on mouse tumor cells *in vitro* involves an extensive inhibition of respiration and of aerobic and anaerobic glycolysis. This action can be counteracted by preincubating the serum with certain polysaccharides or salts and can be enhanced by preincubating the serum with small amounts of distilled water. The cytotoxic action is highly dependent on temperature and involves optimal concentrations of Ca^{++} and/or Mg^{++} above and below which cytotoxicity is submaximal. The metabolic effects are closely correlated quantitatively and kinetically with morphologic cellular changes.—J. Nat. Cancer Inst. 26: 681–689, 1961.

THE CYTOCIDAL action of normal serums on neoplastic cells has been studied for over 50 years by many investigators (*e.g., 1–8*). Landy *et al.* (*6, 7*) and Woods *et al.* (*8*) have recently evaluated the lethal effect of serums of many species on various tumor cells of mice. They studied the cytocidal action of serums in terms of microscopic appearance of cells, vital staining, inoculation tests *in vivo*, and glycolytic inhibition as measured manometrically. The experimental components essential for cytocidal action were shown to be active serums, unheated and suitably preserved, containing natural antibody and 4 components of complement (*9*), divalent cations, *e.g.*, Ca^{++} and/or Mg^{++}, and the susceptible cells.

Test systems and techniques.—Glycolytic and respiratory rates were determined at 38° C with standard Warburg manometers and vessels. Respiration was also measured in the newly designed vessels of Warburg and Krippahl (*11*),[5] with a central elevated trough and connected sidearm,

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[4] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

[5] We have observed that when $K_4Fe(CN)_6$ is used in the sidearm or trough, as proposed in (*11*), HCN gas may develop after hours or minutes, depending upon the temperature and, especially, strong light. Such HCN, when formed in sufficient quantity, may inhibit respiration (ca 50% at 10^{-3} M) and induce or increase aerobic glycolysis, in a well-known manner involving the Pasteur effect. The HCN formation may be detected chemically with $AgNO_3$ (producing a white, highly insoluble AgCN) or colorimetrically with Robbie's phenophthalin-$CuSO_4$ reagent (producing a deep red). To avoid formation of the HCN in disturbing quantity even weak illumination of the $K_4Fe(CN)_6$ reagent is to be guarded against, in experiments of several hours' duration.

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to which was added a mixture of 3 parts 3 M $KHCO_3$ and 1 part 3 M K_2CO_3 containing an adequate amount of commercial carbonic anhydrase (1 to 2 mg/cc) pipetted in just before use. This mixture, with adequate shaking in the trough and sidearm, maintained at 38° C a strictly constant physiological pressure of about 5 percent CO_2 in the gas phase, so that any pressure decrease observed represented simply oxygen consumption, after correction against a thermobarometric control vessel containing the same bicarbonate-carbonate-carbonic anhydrase mixture. Measurements of the content of adenosinetriphosphate (ATP) plus adenosinediphosphate (ADP) in the cells were made with the technique and personal collaboration of I. F. Seits (*12*).

In the present metabolic studies we wished to investigate *in vitro:* 1) the cytotoxic effects of serums on respiration as well as aerobic and anaerobic glycolysis, and to correlate the manometric results with those of vital staining; 2) the effect of temperature on time of onset of glycolytic inhibition; 3) the concentrations of Ca^{++} and Mg^{++} necessary for the cytotoxicity; 4) synergistic inhibition of glycolysis by triple-distilled water and active serum, and diminution of cytotoxicity by preincubating active serum with certain salts; and 5) the prevention of cytotoxic action by preincubating serum with bacterial endotoxic polysaccharides, P-25 and P-45, and with polysaccharides prepared from Sarcoma 37 and mouse liver.

MATERIALS AND METHODS

Tumor cells.—Ehrlich and Krebs-2 ascites carcinoma cells were propagated intraperitoneally in Swiss mice. The cells were harvested 6 to 9 days after inoculation, washed 2 times with Krebs-Ringer-bicarbonate-glucose solution at 4° C, and brought to the desired cell concentration in the same medium.

Media.—Human serum pools were prepared by a mixture of equal amounts of serum from each of 15 donors. Aliquots of each pool were dispensed in flame-sealed pyrex tubes, stored at −20° C, and thawed immediately prior to use. Inactive serum was prepared by heating such pooled active serum for 20 minutes at 56° C, to eliminate essential complement components. Standard Krebs-Ringer-bicarbonate solution was prepared according to Umbreit *et al.* (*10*, p. 149). The sodium bicarbonate was ordinarily 0.025 M and the dextrose 0.011 M. The gas phase contained 5 percent CO_2 in N_2, for anaerobic glycolysis or air for aerobic glycolysis.

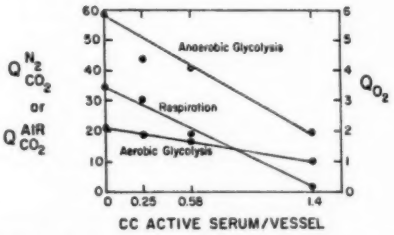
Microscopic evaluation.—Cytotoxic action of serum was also followed by microscopic examination of cells. The rate of observable morphologic changes increased with temperature and concentration of serum. Within a period of some 5 minutes at 38° C, many of the cells became swollen and developed a variable number of cytoplasmic protrusions. The cytoplasm then gave the appearance of being "washed out," and nuclei

became pyknotic. At the same time the cells became stainable with eosin. With sufficient cytocidal agent present, all the cells eventually developed into "ghosts" as the final outcome of the effects initiated by the cytotoxic action of active human serum. One-tenth percent solution of eosin in isotonic saline was used as a vital dye in evaluating the percent killing of cells after a given metabolic run. Eosin penetrated nonviable cells and produced diffuse staining of both cytoplasm and nucleus. Trypan blue staining was also used to corroborate findings obtained with eosin. The staining occurred chiefly in the nucleus and was more marked when applied soon after serum action.

RESULTS

Cytotoxicity Directly Proportional to the Quantity of Serum Added to the Given Cell Population

In text-figure 1 is shown a typical experiment in which stabilized inhibitions of anaerobic and aerobic glycolysis and also of respiration were



TEXT-FIGURE 1.—Respiration and aerobic and anaerobic glycolysis as a function of amount of active serum added; 20 mm³ Krebs-2 ascites cells/total liquid volume, 2.4 cc/vessel.

proportional to the quantity of active serum in a given total volume, 1.4 cc, of active plus inactive serum. At any given concentration of serum, once a given percentage inhibition had attained its full value, the metabolic value remained relatively stable for up to several hours. In the experiment in text-figure 1, the respiration was inhibited somewhat more than the glycolyses, percentage-wise. The ratio of eosin-stained cells to nonstainable cells roughly paralleled the degree of inhibition of respiration or of aerobic or anaerobic glycolysis. The concentrations of energy-rich ATP plus ADP in the cells similarly paralleled the degree of metabolic inhibition (table 1).

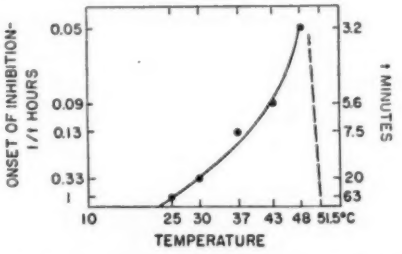
Influence of Temperature on Onset of Glycolytic Inhibition by Human Serum

Inhibition of glycolysis by active serum is a highly temperature-dependent reaction. At 10° C no cytotoxic action was observed over a period of 30 hours. At 25° C 60 minutes were required for observable

TABLE 1.—Measurement of cytotoxicity of increasing amounts of active serum in terms of final glycolytic inhibition and final ATP + ADP concentration

	Active serum (cc)					
	0.0	0.1	0.2	0.3	0.4	0.6
$Q^{N_2}_{CO_2}$, anaerobic glycolysis	65.0	64.0	65.0	61.0	57.0	34.0
ATP + ADP equivalent to μg of P per 75 mm³ of K-2 ascites cells	14	13.6	14.0	12.9	12.2	8.2

onset of glycolytic inhibition; at 30° C, 20 minutes; at 37° C, 7.5 minutes; at 43° C, 5.6 minutes; and at 48° C, 3.2 minutes (text-fig. 2). At 51.5° C, however, cells glycolyzed without inhibition, since the active serum became inactive at this temperature during the equilibration period when the serum was in the vessel sidearm, before it was tipped in with the cells in the main compartment of the vessel.

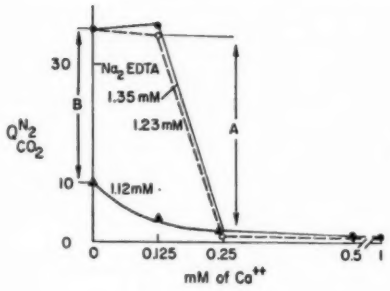


TEXT-FIGURE 2.—The effect of temperature on time of onset of glycolytic inhibition; 8 mm³ Ehrlich ascites cells/total liquid volume, 1.7 cc/vessel; 0.7 cc active serum tipped from sidearm at time t = 0.

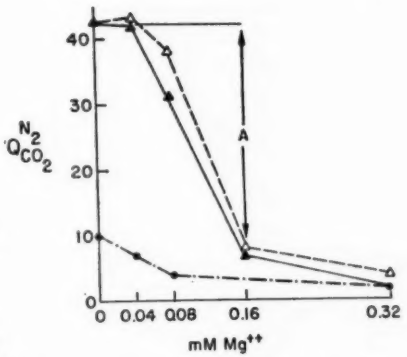
Concentrations of Calcium or Magnesium Ions Necessary for the Cytotoxic Effect

Active serum aliquots, 0.75 cc, in vessel sidearms were incubated with 3 different concentrations of Na_2EDTA (1.12, 1.23, and 1.35 mM), and each concentration of Na_2EDTA at, respectively, 5 concentrations of Ca^{++} (0, 0.125, 0.25, 0.5, and 1 mM, text-fig. 3); or 5 concentrations of Mg^{++} (0, 0.04, 0.08, 0.16, and 0.32 mM, text-fig. 4). The indicated concentrations of Na_2EDTA, Ca^{++}, and Mg^{++} were the final concentrations after the incubated aliquots had been tipped from the vessel sidearms into the main compartments containing 1 cc of cells suspended in calcium-magnesium-free medium (total volume 1.75 cc).

There was no discernible glycolytic inhibition at the two highest concentrations of Na_2EDTA with 0 or 0.125 mM of added Ca^{++} (text-fig. 3); Ca^{++} was presumably highly chelated and unavailable for cytotoxic action. At 0.25 mM of Ca^{++}, however, these 2 concentrations of Na_2EDTA were evidently insufficient to bind all the Ca^{++}, since a strong glycolytic inhibition, up to 97 percent, was observed. On the other hand, when the



TEXT-FIGURE 3.—Glycolytic inhibition curves at 3 different concentrations of Na_2EDTA, each with 4 different concentrations of Ca^{++}; 35 mm^3 Krebs-2 cells/total liquid volume, 1.75 cc/vessel. A) Increase of Ca^{++} concentration from 0.125 to 0.25 mM Ca^{++} increased inhibition of glycolysis from 0 to 97 percent. B) Decrease of Na_2EDTA from 1.23 to 1.12 mM Na_2EDTA increased inhibition of glycolysis from 0 to 75 percent.



TEXT-FIGURE 4.—Glycolytic inhibition curves at 3 different concentrations of Na_2EDTA, as in text-figure 3 (△ 1.35 mM, ▲ 1.23 mM, and ● 1.12 mM), each with 4 different concentrations of Mg^{++}; 35mm^3 Krebs-2 cells/total liquid volume, 1.75 cc/vessel. A) Increase of Mg^{++} concentration from 0.04 to 0.16 mM Mg^{++} increased inhibition of glycolysis from 0 to 83 percent.

Na_2EDTA concentration was reduced from 1.23 mM to 1.12 mM, with no added Ca^{++} or no added Mg^{++}, the glycolysis was inhibited 75 percent since some serum Ca^{++} was then left free for cytotoxic action. Likewise, there was no glycolytic inhibition at the 2 highest concentrations (1.35 and 1.23 mM) of Na_2EDTA with 0 or 0.04 mM of Mg^{++} (text-fig. 4). However, at 0.16 mM of Mg^{++}, as with 0.25 mM of Ca^{++}, glycolysis was sharply inhibited (83%). At 0.16 mM of Mg^{++}, some Mg^{++} was left free for cytotoxic action. Clearly, Ca^{++} and Mg^{++} are each effective in cytotoxic action at roughly the same orders of concentration.

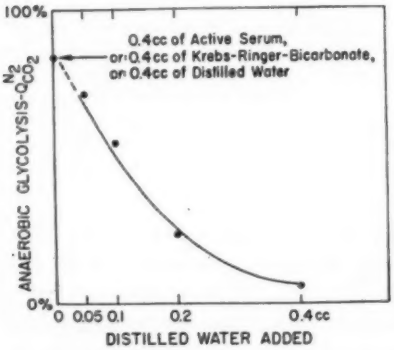
We have thus approached the quantification of Ca^{++} and/or Mg^{++} concentrations either by an increase in Na_2EDTA concentration in serum or have overcome its effect by known amounts of added ions. Both approaches gave us comparable stoichiometric and glycolytic inhibition results. On the other hand, as the concentrations of either Ca^{++} or Mg^{++} are raised above a certain limit, an optimum effect is passed through and their action is reversed, with increasing prevention of serum toxicity.

Synergistic Inhibition of Glycolysis by Triple-Distilled Water Serum, and Diminution of Cytotoxicity by Coincubating Active Serum With Certain Salts

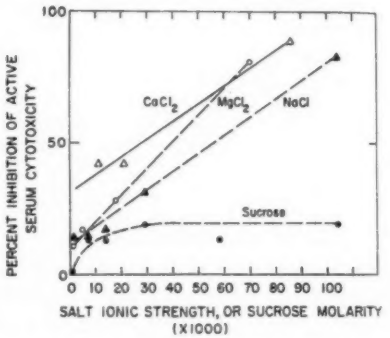
We found it possible to increase considerably the cytotoxic action of limiting amounts of active serum by addition of small amounts of distilled water. An extreme example is shown in text-figure 5, where the ratio of active serum to tumor cells was reduced to a point at which no

cytotoxic action was observable with either serum or distilled water added separately. However, when 0.4 cc serum aliquots were first coincubated in the vessel sidearms with variable amounts of distilled water (as little as 0.05 up to 0.4 cc), and then tipped into the main vessel compartments containing tumor-cell suspensions (total volume 1.8 cc), striking synergistic inhibition of glycolysis was observed. The effect could be either minimized with isotonic concentrations, or completely abolished with hypertonic concentrations, by adding to the distilled water varying amounts of NaCl, and/or $MgCl_2$, and/or $CaCl_2$.

Furthermore, a proportional loss of serum cytotoxicity was observed when variable amounts of NaCl, $MgCl_2$, $CaCl_2$, or $SrCl_2$ were first coincubated with active serum before tipping into the main vessel compartment containing tumor cells. On an equimolar basis sucrose was much less effective than the salts (text-fig. 6).



TEXT-FIGURE 5.—Synergistic inhibition of glycolysis of Ehrlich ascites cells induced by distilled water and active human serum; 75 mm³ Ehrlich ascites cells/total liquid volume, 1.8 cc/vessel. Each vessel contained 0.4 cc active serum and variable amounts of distilled water up to 0.4 cc with Krebs-Ringer-bicarbonate medium from 1.0 to 1.4 cc.

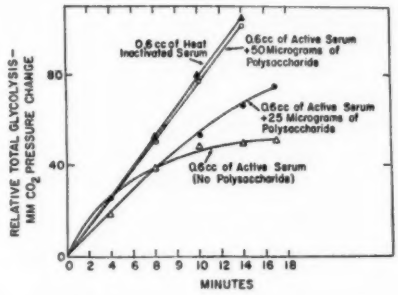


TEXT-FIGURE 6.—Inhibition of serum cytotoxicity by increasing concentrations of $CaCl_2$, $MgCl_2$, NaCl, and sucrose; 35 mm³ Krebs-2 cells/total liquid volume, 1.8 cc/vessel.

Effect of P-25 and P-45 Endotoxic Polysaccharides and of Polysaccharides Prepared From Sarcoma 37 and Mouse Liver

We found that the cytotoxic action of active serum can be either decreased or completely abolished if serum is first incubated at 38° C for 30 minutes with different amounts of bacterial endotoxic polysaccharides, P-25 and P-45, or with polysaccharides prepared from Sarcoma 37 or mouse liver. Eighty to 100 μg of Sarcoma 37 and mouse-liver polysaccharide preparations abolished completely the cytotoxic action of 1 cc of active serum; twice that amount of P-25 or P-45 was required. Fifty μg of the polysaccharide from Sarcoma 37 completely abolished

the cytotoxicity of 0.6 cc active serum, after their coincubation for 30 minutes in a vessel sidearm before it was tipped into the main vessel compartment containing the tumor-suspension cells (text-fig. 7). Glycolysis obtained after this mixture was tipped equaled the glycolysis obtained after 0.6 cc of heat-inactivated serum as control was tipped into the compartment. By the end of the metabolic run, 0.6 cc of active serum alone almost completely abolished glycolysis.


RELATIVE TOTAL GLYCOLYSIS – MM CO_2 PRESSURE CHANGE
80
40
0
0 2 4 6 8 9 10 12 14 16 18
MINUTES
0.6 cc of Heat Inactivated Serum
0.6cc of Active Serum +50 Micrograms of Polysaccharide
0.6cc of Active Serum +25 Micrograms of Polysaccharide
0.6cc of Active Serum (No Polysaccharide)

TEXT-FIGURE 7.—Inhibition of serum cytotoxicity by polysaccharide preparation from Sarcoma 37; 30 mm^3 Ehrlich ascites cells/total liquid volume, 1.6 cc/vessel.

The amounts of polysaccharides necessary for inactivating cytotoxicity closely paralleled the anticomplement effect of these compounds as independently measured by immune hemolysis tests carried out with sheep red blood cells, active human serum, and hemolysin.

CORRELATIVE STUDIES AND DISCUSSION

Metabolic studies carried out in conjunction with microscopic observations and vital staining techniques provide a more accurate, though still imperfect, resolution of the cytotoxic serum effect. Indications, not yet conclusive, are that the individual cells respond to active serum in a virtually all-or-none manner; this view is expanded more fully by Landy *et al.* (*7*). Thus, with increasing amounts of serum added to a given cell population, there is a rough proportionality between inhibition of glycolysis or respiration of the cell population and number of eosin-stained "dead" cells. In addition, in the total cell population, those cells remaining impermeable to eosin apparently continued to metabolize for hours without appreciable deterioration, whereas the stained cells underwent rather rapid and marked morphologic changes suggestive of extensive intracellular hydrolysis. Green *et al.* (*13*) have made quantitative analyses of such various chemical changes in a comparable study involving an immune complement-dependent system.

Several glycolytic and respiratory substrates were tested to determine whether they could support either glycolysis or respiration. The results

were negative, except with succinate, which permitted a rate of respiration that was normal quantitatively (equal to the inactivated serum control) though probably not normal qualitatively. Although such a high support of respiration indicates intactness of the enzymes involved in succinate metabolism (*14*), such a high support also indicates extensive cellular damage, according to Woods (*15*).

Bickis *et al.* (*16*) reported that, in an immune complement-dependent system, glycine uptake by the Ehrlich ascites cells was impaired even before microscopic changes were observable. It would be of interest here to know whether the nonkilled cells would show inhibition of glycine uptake at the point when there is no measurable respiratory or glycolytic inhibition.

The most interesting new observations in the present study were the effect of even small amounts of distilled water in promoting the over-all cytotoxic action, and the effect of certain endotoxic and other polysaccharides in inhibiting the cytotoxic action.

There are several possible explanations for the synergistic cytotoxic action of small amounts of distilled water and serum. Hypotonicity, even small, might increase complement effectiveness. Becker and Wirtz (*17*) have demonstrated a "salt-sensitive" step in immune hemolysis: hypotonicity increasing the efficiency of hemolysis, whereas hypertonicity had the reverse effect. In the present studies a diminution of cytotoxic effect was also similarly obtained with hypertonicity. It may well be that the same type of mechanism is involved, especially if one takes into account other similarities between the hemolytic immune system and the present one involving "natural antibody." Hypotonicity may cause cellular swelling and possible leakage of cellular constituents, or cellular swelling by itself might expose new antigenic sites which, if involved in the cytotoxic reaction, might prove more injurious to the cell. On the other hand, the additive effect of distilled water may be all that is necessary for the "subliminally injured" cells to succumb. It is to be emphasized that an amount of distilled water, as little as 0.05 cc in 1.8 cc total volume, had a noticeable inhibitory effect on anaerobic glycolysis.

Since there is a strong parallelism of anticytotoxicity and anticomplementary properties of the tested polysaccharide compounds, one is tempted to explain the anticytotoxic effect as due to the anticomplement effect of these compounds. In further work it would be of great importance to determine which of the component(s) of the complement is (are) inactivated by the polysaccharides used, or whether polysaccharide preparations employed might be interacting directly with the serum "natural antibodies." In this connection numerous efforts were made to see whether polysaccharides showed competitive inhibition predominantly against complement or predominantly against antibody, but our efforts failed because of inability to obtain complement sufficiently free of antibody and antibody sufficiently free of complement to provide adequate competitive inhibition analyses and hence adequate specificity of conclusion.

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Chinese-Hamster Cell Strains *In Vitro*: Spontaneous Chromosome Changes and Latent Polyoma-Virus Infection [1, 2]

DENYS K. FORD, CHRISTINA BOGUSZEWSKI, *and* NELLY AUERSPERG,[3] *Department of Medicine and G. F. Strong Laboratory for Medical Research, University of British Columbia, Vancouver, B. C., Canada*

SUMMARY

The spontaneous changes found in the chromosome complements of 35 Chinese-hamster cell cultures are described. Unpredictable variation in chromosome count was a characteristic feature, and, after 1 to 2 years *in vitro*, from 10 to 30 percent of the chromosomes were aberrant. Cell cloning and improvement of the culture media failed to stabilize significantly the chromosome constitution of the cultures. Eleven sublines were inoculated with the SE polyoma virus, but it was not possible to demonstrate specific effects of the virus on the chromosome complements of the infected cultures. One inoculated subline liberated infective polyoma virus into the surrounding media continuously for 282 days, though the cultures looked healthy and proliferated normally.—J. Nat. Cancer Inst. 26: 691–706, 1961.

PREVIOUS REPORTS (*1*, *2*) have outlined spontaneous changes occurring in the chromosomes of Chinese-hamster cell strains grown for 12 to 22 months in tissue culture. The investigation has been continued, and this paper describes observations on cell strains cultured *in vitro* for up to 3 years, and also observations on cell clones. In addition, cell strains have been inoculated with the SE polyoma virus to determine whether infection with this agent will induce chromosome changes in tissue culture suggestive of a direct effect of the virus on the genetic mechanism of the cell.

MATERIALS AND METHODS

The previously described Chinese-hamster cell strains C, P, V, and X were maintained in duplicate sublines and further observed; moreover, 25 new strains were initiated from trypsinized cell suspensions, 13 being from the lungs and 9 from the kidneys of adult animals. The tissue-culture

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methods and procedures were similar to those previously reported (*1*) with the following alterations over the past year. NCTC medium 109, obtained from Difco, Detroit, Michigan, was used instead of Connaught Laboratories medium 1066. Bovine fetal serum, obtained from the Colorado Serum Laboratories, Denver, Colorado, was employed as a 20 percent supplement, instead of calf serum, to the synthetic medium in culturing most of the new strains. Stock cultures were maintained with success and greater convenience in 2-ounce, soft glass prescription bottles instead of pyrex-glass, milk dilution bottles. The established air-dried coverslip methods (*3*, *4*) were adapted for chromosome preparations, and the cultures were thus grown in Leighton tubes containing coverslips for the purpose of chromosome sampling, the improved preparations resulting in greater precision during the last year of the study.

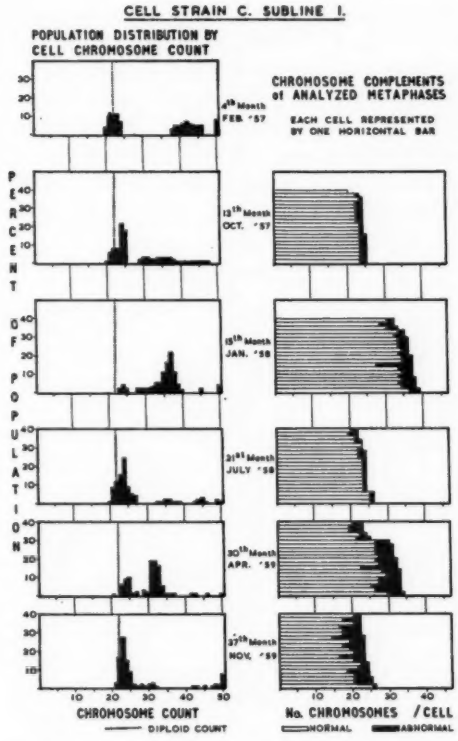
The single-cell isolation technique of Sanford (*5*) was selected for the initiation of cell clones and the procedure carefully checked throughout with microscopy to establish the certainty of single-cell isolation.

One frozen and 2 lyophilized samples of the SE polyoma virus were received from Dr. Bernice Eddy, of the Division of Biologic Standards, National Institutes of Health, Bethesda, Maryland. All work with the virus was performed in an isolated laboratory, and all materials leaving this laboratory were autoclaved or immersed in Wescodyne disinfectant; moreover, the individual who handled the cultures used a gown, glove, and mask technique. The virus was demonstrated by its cytopathogenic effect on mouse-embryo cultures grown in synthetic medium supplemented with 2 percent inactivated horse serum. For this purpose, 3 to 5 days after a medium change, 0.1 to 0.2 ml of tissue-culture medium from the hamster cultures was added to the mouse-embyro cultures and the cytopathogenic effects were observed 6 to 12 days later.

RESULTS

Spontaneous Changes in Stock Cultures

To report the patterns of chromosome behavior in the studied cell strains, histograms are used. Text-figures 1 to 9 outline the changes observed in the C, P, V, X, and Y cell strains, and duplicate sublines are represented separately. In each text-figure the histograms on the left show the population distribution of the cultures by cell chromosome count, while the diagrams on the right show the numbers of normal and abnormal chromosomes in the metaphases that were analyzed; each metaphase is represented by one horizontal bar. The metaphases that were analyzed were selected for their precise chromosome definition, and, except for one occasion, only metaphases with diploid to tetraploid numbers were analyzed. The cells were also selected so that their chromosome counts had a distribution approximately similar to that of the population distribution. In each of the population-distribution histograms the normal

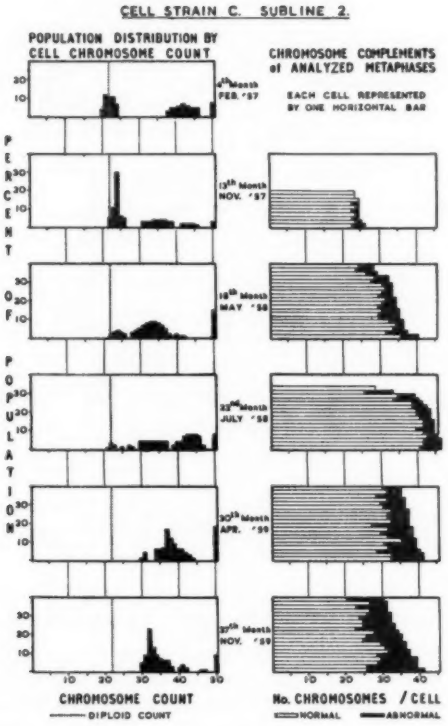


TEXT-FIGURE 1.—Population distribution by cell chromosome count and incidence of abnormal chromosomes in successive observations of cell strain C, subline 1.

diploid count of 22 is shown by a dotted line, and the percentage of cells shown to have a count of 50 includes those in which the count was more than 50.

The sequence of events in the C strain is illustrated by text-figures 1 and 2. Thus about half the population of the C-1 subline was in the diploid region during the 1st year (text-fig. 1) but subsequently in the 15th month the culture became predominantly hypertriploid with the most common count being 37. There was then a shift to the diploid region by the 21st month, but by the 30th month, cells with counts of 31 to 33 predominated. Finally, in the 37th month the most frequent count was 23, but most of these "diploid" metaphases had about 5 aberrant chromosomes. The C-2 subline (text-fig. 2) showed a broad spread of chromosome counts in the 22 to 40 range in the 18th month of culture. By the 30th month the majority of cells were in the 35 to 45 region with a peak at 37, but when last examined in the 37th month the predominant pattern had shifted to the 30 to 35 count, of which usually 6 to 8 chromosomes were aberrant.

The P-1 subline (text-fig. 3) remained predominantly diploid for the 35-month observation period with the exception that the culture had cells with complements throughout the diploid, triploid, and tetraploid ranges in the 14th month. When last examined in the 35th month most of the cells were "diploid" but contained 3 to 4 aberrant chromosomes per cell. The P-2 subline (text-fig. 4) was "diploid" for the 1st year in culture but then showed a variable pattern culminating in the majority of cells having counts of 47 to 56 in the 35th month with the peak number at 51; approxi-



TEXT-FIGURE 2.—Population distribution by cell chromosome count and incidence of abnormal chromosomes in successive observations of cell strain C, subline 2.

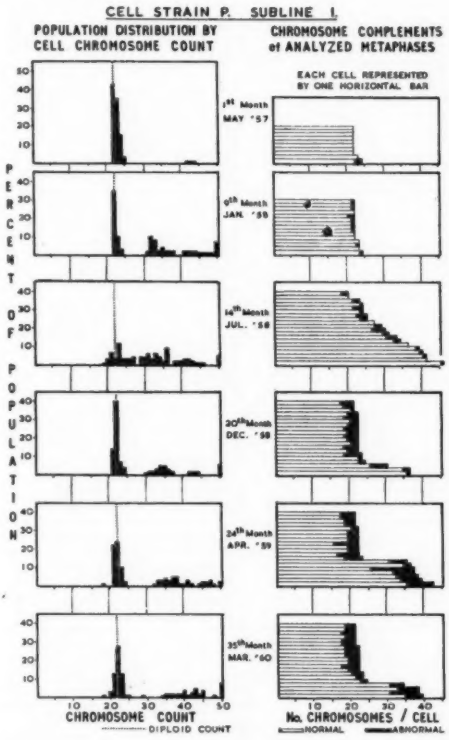
mately 30 percent of the chromosomes of these hypertetraploid cells were aberrant.

Both sublines of the V strain (text-figs. 5 and 6) remained "diploid" during the 29 months of observation, but terminally over 20 percent of the chromosomes were aberrant in subline 1 and about 15 percent in subline 2.

The sublines of the X strain (text-fig. 7) were observed for 21 and 22 months, and in both there was considerable deviation from normal diploidy and normal chromosome morphology.

Strain Y (text-fig. 8) was observed for only 11 months and subline 1 retained a "diploid" complement, whereas subline 2 in the 11th month contained cells with widely differing chromosome counts.

Two sublines of V-clone-3 are illustrated in text-figure 9. The results of cloning the V strain will be discussed more fully, but the varied chromosome complements of this clone in the 4th and 6th months after single-cell isolation are evident. Subsequently both sublines became more uniform in the hyperdiploid region.

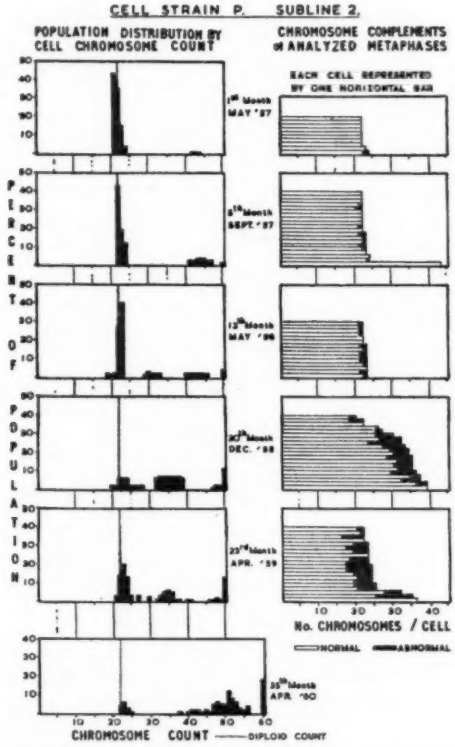


TEXT-FIGURE 3.—Population distribution by cell chromosome count and incidence of abnormal chromosomes in successive observations of cell strain P, subline 1.

The most striking feature of the study, therefore, was the variability of the chromosome constitution between different cell strains, between sublines of the same strain, and between successive preparations from the same subline. Only the V cell strain remained quite normal for longer than the first 3 to 4 months after initiation of the culture, and the V-2 subline retained a predominantly normal diploid complement for the first 10 months of life *in vitro* (text-figs. 5 and 6).

Whereas the most common chromosome count was usually 22 to 25, the modal chromosome counts of the cultures varied from 21 to 51. Thus

the C sublines showed modal counts of 31 and 37 (text-fig. 1), and 32 (text-fig. 2); the P-2 subline had a modal count of 51 in the 35th month (text-fig. 4), the X-2 subline modal counts of 26 to 27 (text-fig. 7), and the V-clone-3, subline 2 a majority of the cells in the 37 to 42 range of chromosome number (text-fig. 9). In most cultures an appreciable number of metaphases had twice the modal number of chromosomes; thus the "diploid" cultures contained "tetraploid" cells and the "triploid" cultures had some metaphases in the 60 to 70 range, while the P-2 subline

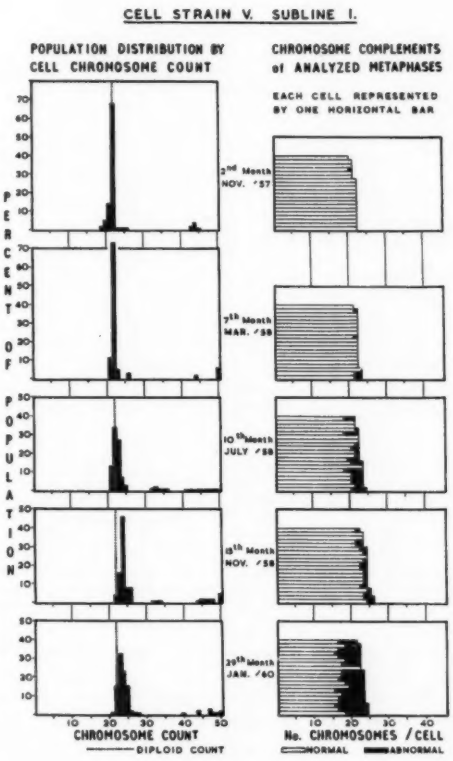


TEXT-FIGURE 4.—Population distribution by cell chromosome count and incidence of abnormal chromosomes in successive observations of cell strain P, subline 2.

in the 35th month, with a majority of the cells in the 47 to 54 range, had an appreciable proportion of metaphases with counts approximating 100.

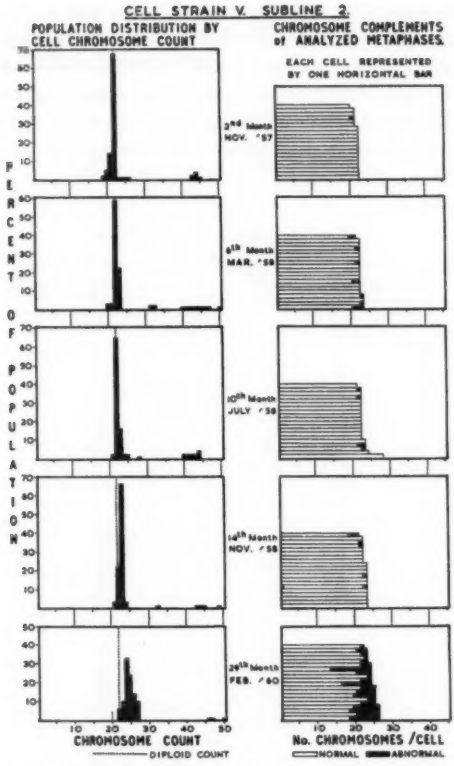
Although hyperdiploidy was the commonest chromosomal pattern, there was no uniform tendency in these cultures for the cells to have progressively increasing numbers of chromosomes as their life *in vitro* continued. Moreover, a predominant complement with a high chromosome count could be replaced by a complement with a lower count. The C-1 subline, between the 15th and 21st months, and both the V-clone-3 sublines

showed a shift in population from cells with counts in the upper 30's to cells with near-diploid counts. However, a longer period of culture did result in increasing numbers of abnormal chromosomes, as shown in the diagrams on the right of the text-figures. Whereas aberrant chromosomes were rare initially, old cultures showed up to 25 percent of abnormal forms, as found in the near-diploid V-1 subline in the 29th month (text-fig. 5).



TEXT-FIGURE 5.—Population distribution by cell chromosome count and incidence of abnormal chromosomes in successive observations of cell strain V, subline 1.

Detailed observations on the types of aberrant chromosomes in these cultures were not made beyond those already described (*2*) and the opinions expressed previously have not been substantially altered, though new aberrant telocentric, subterminal, and submetacentric chromosomes of varying sizes have been observed after longer life *in vitro*. The origin of these abnormal forms was not elucidated and no detailed study of the normal and abnormal morphology of the #8 sex chromosomes was undertaken.

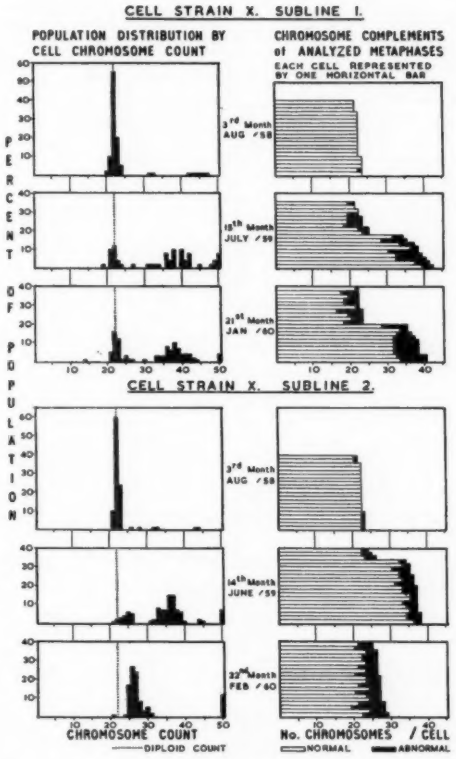


TEXT-FIGURE 6.—Population distribution by cell chromosome count and incidence of abnormal chromosomes in successive observations of cell strain V, subline 2.

Spontaneous Changes in Clone Cultures

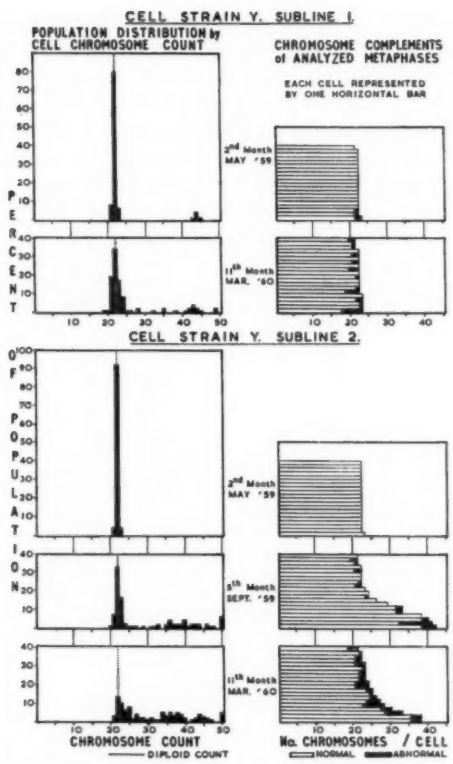
Single-cell isolation was performed on the V-2 subline in its 14th month of life *in vitro*, at a time when the culture was near-diploid. Three clones were established in continuous culture and detailed studies were made on clones 1 and 2, but clone 3 was not investigated as closely because, when first examined in the 4th month after single-cell isolation, the culture was composed of cells with variable complements in the 30 to 45 range of chromosome number. Clones 1 and 2 had fairly uniform near-diploid complements and the sequence of events in these cultures is illustrated in text-figures 10 and 11. In the diagrammatic representation of the analyzed cells to the right of these text-figures, the number of chromosomes in each group of morphologically similar chromosomes is shown separately: (*a*) the group of large metacentric #1 and 2 chromosomes, (*b*) the medium-sized metacentric or submetacentric #3, 4, and 8 chromosomes, (*c*) the small metacentric #5, 6, and 7 chromosomes, (*d*) the subterminal #9, 10, and 11 chromosomes, and (*e*) the abnormal chromosomes. Within

the group of small #5, 6, and 7 chromosomes there has been included a small subterminal chromosome which sometimes could be distinguished only with difficulty from a #7 chromosome; in the text-figures these have been labeled "S" in a subcategory of the #5, 6, and 7 group. In clone 1 the initial complement was relatively uniform with a count of 23 and was composed of the normal complement without one of the medium-sized metacentrics and with the addition of both an extra small #7 or "S"



TEXT-FIGURE 7.—Population distribution by cell chromosome count and incidence of abnormal chromosomes in successive observations of cell strain X, sublines 1 and 2.

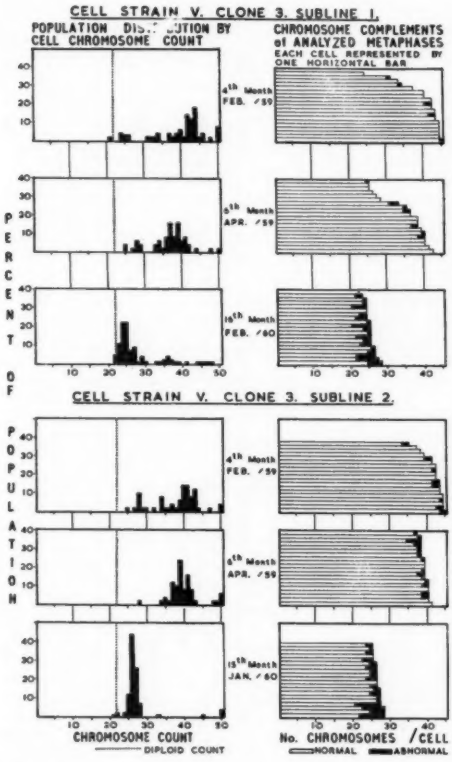
chromosome and 1 telocentric. Clone 2 when first examined in its 4th month had 2 predominant patterns which differed slightly from each other and from clone 1. The loss of uniformity in the later diagrams of text-figures 10 and 11 indicates the variable complements of the cultures, clone 1 becoming irregular by the 10th month and clone 2 by the 8th month. The cloning of mixed cell strains, therefore, did not significantly reduce the variability of the chromosome complements of these hamster cultures.



TEXT-FIGURE 8.—Population distribution by cell chromosome count and incidence of abnormal chromosomes in successive observations of cell strain Y, sublines 1 and 2.

Failure of Fetal Serum to Prevent Spontaneous Changes

To determine more precisely the length of time over which new cultures might remain diploid and to compare the effects on chromosome stability of calf serum with fetal calf serum as a 20 percent medium supplement, 24 cultures were initiated from 7 animals and observed until they had lost the normal diploid complement or up to March, 1960, when the investigation was terminated. Table 1 gives the result of this experiment and shows that more cultures were observed on 20 percent fetal serum than on the calf serum because the former was more successful in initiating new cultures. In the first 2 months, 9 of 13 cultures on fetal serum and 4 of 6 cultures on calf serum maintained a predominantly normal diploid complement. In the 3d and 4th months, 4 of 11 cultures on fetal serum and 3 of 4 on calf serum retained predominantly normal diploidy. In the 5th and 6th months none of the examined cultures on either serum demonstrated a predominantly normal diploid complement. It seemed,

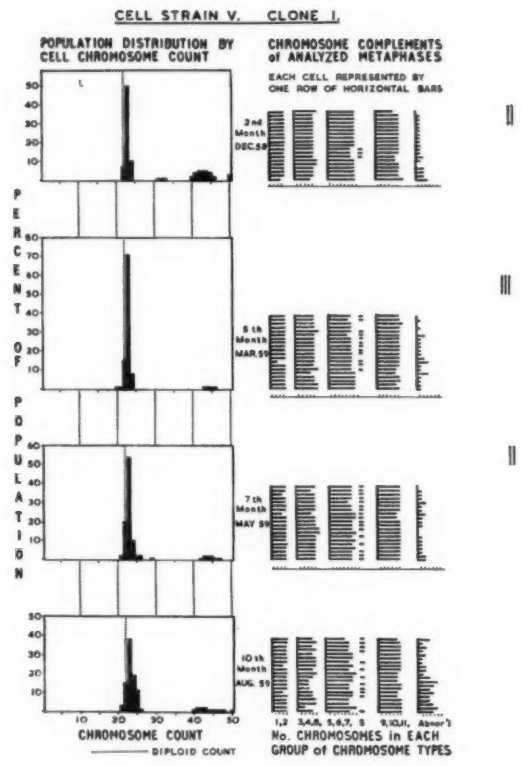


TEXT-FIGURE 9.—Population distribution by cell chromosome count and incidence of abnormal chromosomes in successive observations of cell strain V, clone 3, sublines 1 and 2.

therefore, that fetal serum did not increase the chromosome stability of the cultures, though it did achieve greater success in starting new cultures from trypsinized lungs and kidneys. Table 1 also indicates that the percentage of cells with normal diploid complements was often less than that of cells with counts of 22. In some instances the difference was marked as in E lung on fetal serum and D lung on calf serum. This observation once again emphasizes the limited value of simple chromosome counts without analysis of the complement for morphological changes in individual chromosomes.

Observations on Cultures Infected With Polyoma Virus

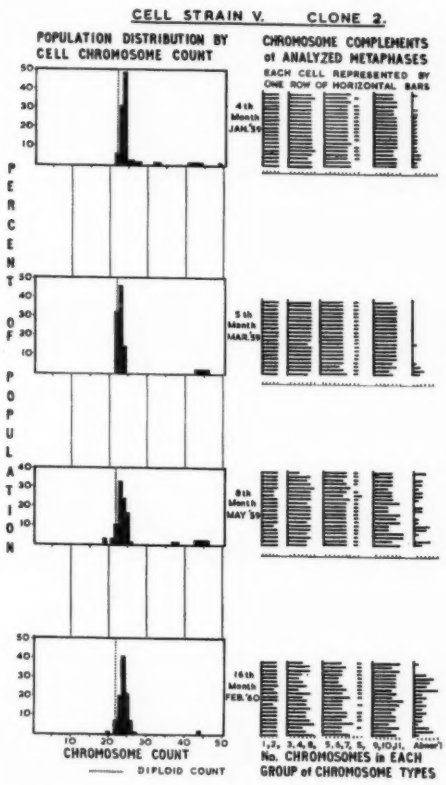
Eleven hamster-cell sublines were infected with the SE polyoma virus obtained from Dr. Bernice Eddy. The sublines were grown in media supplemented with 2 percent inactivated horse serum for the 1st week after infection; this was increased to 5 percent horse serum for the 2d


CELL STRAIN V. CLONE 1.
POPULATION DISTRIBUTION BY CELL CHROMOSOME COUNT
CHROMOSOME COMPLEMENTS of ANALYZED METAPHASES
EACH CELL REPRESENTED BY ONE ROW OF HORIZONTAL BARS
PERCENT OF POPULATION
2nd Month DEC. 58
5th Month MAR. 59
7th Month MAY 59
10th Month AUG. 59
CHROMOSOME COUNT
DIPLOID COUNT
No. CHROMOSOMES in EACH GROUP of CHROMOSOME TYPES

TEXT-FIGURE 10.—Population distribution by chromosome count and chromosome complements of analyzed metaphases in successive observations of cell strain V, clone 1.

week and thereafter the cultures were returned to their routine media, 10 percent calf serum for the clone 1 and Y cultures and 20 percent fetal serum for the D cultures. For each infected subline a control subline was treated in an exactly similar manner and observed an equal time. These control cultures were tested for the presence of mouse-embryo cytopathogenic agents at the same time as the inoculated cultures and no cytopathogenic changes were ever observed. The chromosome complements of infected and control cultures were studied at intervals to determine whether polyoma-virus infection caused chromosome aberrations. The results of the mouse-embryo cytopathogenicity tests and the chromosome observations are shown in table 2.

By mouse-embryo cytopathogenicity tests it was demonstrated that each infected hamster-cell subline was inoculated with viable virus, and within the 1st few weeks of infection the supernatant media from the Y and D sublines were tested and found to contain virus. The clone 1 sublines were not releasing virus when tested on the 84th day after inocula-


CELL STRAIN V. CLONE 2.
POPULATION DISTRIBUTION BY CELL CHROMOSOME COUNT
CHROMOSOME COMPLEMENTS of ANALYZED METAPHASES
EACH CELL REPRESENTED BY ONE ROW OF HORIZONTAL BARS
PERCENT OF POPULATION
4th Month JAN.'59
5th Month MAR.'59
8th Month MAY '59
16th Month FEB.'60
CHROMOSOME COUNT
DIPLOID COUNT
No. CHROMOSOMES in EACH GROUP of CHROMOSOME TYPES

TEXT-FIGURE 11.—Population distribution by chromosome count and chromosome complements of analyzed metaphases in successive observations of cell strain V, clone 2.

tion. On the other hand, the Y-1 culture released virus for 74 days and the Y-2 subline for the whole of the 282-day observation period. Neither of these cultures showed any cytopathogenic changes while grown in their routine 10 percent calf-serum medium and their growth rate appeared comparable to that of the controls. Repeatedly from the 129th to the 282d day, supernatant medium from the Y-2 subline caused marked cytopathogenic changes in mouse-embryo cultures, though there were no morphological or cultural differences between the subline and its control. This latent infection of the Y-2 subline seemed of interest and living samples of the cells were sent to Dr. Bernice Eddy for confirmation of polyoma-virus release. Dr. Eddy demonstrated by mouse-embryo cytopathogenicity, hemagglutination, and tumor induction in Syrian hamsters with inhibition by specific antipolyoma serum that the polyoma virus was present in this culture over 200 days after inoculation with the virus. In her laboratory the subline showed degenerative changes when

TABLE 1.—Comparison of fetal serum with calf serum on the chromosome stability of new cell strains

Culture	Percentage of cell population								
	1 to 2 months			3 to 4 months			5 to 6 months		
Cell strain (fetal serum)	Near-diploid count	Count 22	Normal diploid complement	Near-diploid count	Count 22	Normal diploid complement	Near-diploid count	Count 22	Normal diploid complement
Z-l*				96	91	86	96	77	35
D-l	94	94	89	96	82	67	96	38	29
D-k*	86	57	53	82	49	37			
E-l	92	92	28	86	77	26	86	9	0
E-k	94	89	85	98	64	39	—	—	—
E-o*	100	100	85	94	14	14	—	—	—
E-m*	98	88	88	94	47	47	—	—	—
G-l	100	95	85	98	83	69	—	—	—
G-k				90	50	32	—	—	—
H-l	96	86	76	96	62	62	—	—	—
H-k	94	66	66	96	45	40	—	—	—
I-l	98	25	25	—	—	—	—	—	—
I-k	100	10	10	—	—	—	—	—	—
K-l	100	100	95	—	—	—	98	59	34
K-k	86	26	13	—	—	—	—	—	—
Cell strain (calf serum)									
D-l	93	70	33	—	—	—	—	—	—
D-k	—	—	—	60	10	10	—	—	—
E-l	96	91	91	92	64	55	—	—	—
E-m	—	—	—	—	—	—	100	30	10
G-l	98	93	83	100	90	75	—	—	—
G-k				98	93	88	—	—	—
I-l	96	90	90	—	—	—	96	50	50
I-k	100	13	7	—	—	—			
K-l	94	61	52	—	—	—	94	19	14

*L = lung, k = kidney, o = ovary, m = muscle fascia.

TABLE 2.—Observations on virus release and chromosome complements of polyoma-infected cell strains

Cell strain	Virus stock	Virus release		Chromosome complements	
		Time after infection (days)	CPC in embryo cultures	Time after infection (days)	Difference from control
Cl-1	A	84	0	38	0
	B	84	0	45	0
	C	84	0		
Y-1	A	45	+++	26	0
		59	+	54	0
		74	++	125	0
		152	0		
		230	0	263	0
Y-2	B	64	+++	36	0
		78	+	88	0
		93	+++	129	0
		171	+++		
		233	+++		
		282	+++	282	?
D (6 separate sublines)	B	12	+++		
		142	0	121	0

grown on synthetic medium supplemented only with 2 and 5 percent horse serum, but on the 10 percent calf-serum supplementation used routinely in this laboratory easily demonstrable virus release was associated with healthy-looking continuous proliferation.

The last two columns of table 2 give the times after polyoma-virus inoculation when the chromosome complements were studied and the results of comparing the infected cultures with the controls. The Y-2 culture on the 282d day was the only strain to show differences between the infected and control sublines. However, the degree of difference was no greater than might be expected to occur spontaneously in 2 sublines of a single strain cultured separately for 282 days; also the Y-2 infected subline showed no distinctive or unique chromosomal features but rather the types of abnormalities that have been commonly observed in stock hamster sublines; moreover, the stock Y-2 culture showed an even more aberrant pattern than the infected Y-2. Thus, although the only culture showing chromosome complements different from those of its respective control was the latently infected Y-2 subline, there was no evidence to suggest that this was a specific effect of the polyoma virus.

DISCUSSION

From the experience obtained during the study of many cell strains and sublines over a 3-year period it would seem inadvisable to make generalizations on the expected chromosomal behavior of hamster-cell cultures *in vitro*. The chromosome complements probably vary continuously and neither cloning nor media improvement stabilizes the chromosome patterns to any significant degree. However, the closer study of the sex chromosomes of Chinese-hamster cells may, as indicated by Yerganian (*6*), reveal

new and more useful data relating chromosome constitution to changes in cell behavior. Unfortunately it was not possible by the methods employed to demonstrate that the polyoma virus acted on the genetic mechanism of the cell to cause chromosome aberrations.

The finding that the polyoma-infected Y-2 subline would continuously and apparently indefinitely release virus without showing any cytopathogenic changes was interesting. "Latent" virus infection has been defined in several ways and may occur under various circumstances. The type of latent infection described here was not extensively investigated in this study and the inhibitory effects of calf serum on the polyoma virus were not evaluated; nevertheless the fact remains that healthy, proliferating cultures were continuously releasing infective virus particles into the surrounding medium.

At the start of the investigative program on cultured Chinese-hamster cells we planned to implant cultures routinely into cheek pouches to study sarcomatous transformations. Forty-two cheek pouches were implanted with 1 to 2 million C subline cells grown 16 to 20 months *in vitro*, but no tumors arose during observation periods up to 19 months. Later, in view of the only partial inbreeding of the animal strains and more particularly because of the continuous chromosome variations, this aspect of the study was abandoned. It seemed unlikely that the methods used in this investigation could lead to meaningful correlations between chromosome patterns and malignant transformations.

At no time during the investigation did the cultures undergo significant morphological or cultural cell alterations in spite of the continuous chromosome changes. Minor variations in cell morphology and growth rate were found, but no dramatic "cell alterations" occurred and the cultures remained "fibroblastic" throughout. After the initial experience of cross-contamination with a human cell strain as described earlier (*1*), the rigorous laboratory procedures instituted to prevent the recurrence of such an event appeared successful. Experience with Chinese-hamster cultures, therefore, supports the view that "cell alterations" should be regarded with suspicion.

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Chromosomal Studies on Diethylstilbestrol-Induced Testicular Tumors in Mice [1, 2]

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SUMMARY

Chromosome cytological studies have been made on three transplanted interstitial-cell tumors of the testis, induced in (A/Sn × A.SW)F_1 mice by estrogen treatment. All three tumors were estrogen-dependent and grew only in females and diethylstilbestrol-treated males, but not in untreated males. Two independent sublines were also examined, which could grow in untreated males and had been isolated from one dependent tumor line. All three dependent tumors were chromosomally changed with stem lines containing 53, 47, and 52 chromosomes, respectively. In addition, some tumors had secondary stem lines with 40 chromosomes, possibly representing myelopoietic cells. There was a considerable variability around the stemline number in all tumors, with a tendency to diminish on serial transplantation. No additional chromosomal changes could be detected in the transition of one dependent tumor line to autonomy. The occurrence of heteroploid modal numbers in the dependent tumors indicates that they consisted of genetically altered cells.—J. Nat. Cancer Inst. 26: 707–717, 1961.

PROLONGED DISTURBANCES of endocrine balance often lead to tumors dependent for their growth on the changed hormonal environment. Sooner or later most dependent tumors tend to become autonomous, *i.e.*, acquire the ability to grow in isologous hosts with a normal hormonal environment (*1*). According to Furth, dependent tumor cells "resemble normal cells and respond fully to physiological stimuli" (*2*). The mechanism of the transition from dependency to autonomy is not known but "is best conceived as a mutation" (*2*).

The present investigation is a karyological study of estrogen-induced, interstitial-cell testicular tumors of the mouse (*1*, *3–5*), carried by serial transplantation in isologous hosts. An attempt was made to establish whether chromosomal abnormalities can be demonstrated in dependent tumors, and whether the development of autonomy is reflected by detectable chromosomal changes. Chromosomally altered stem lines in

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dependent tumors would indicate that they differ from normal cells, since several studies have critically shown that normal somatic mouse cells are strictly euploid (*6–8*).

If chromosomal changes could be regularly detectable in connection with the progression of hormone-dependent tumors to autonomy, they would indicate a genetic mechanism. However, the absence of any chromosomal change cannot exclude genetic changes, since minor chromosomal rearrangements, point mutations, etc., cannot be observed with cytological techniques.

Shifts of karyotype have been described concurrently with other types of phenotypic alterations in tumor-cell populations, such as changes in homotransplantability or sensitivity to certain antimetabolites (*9*). Since the populations used were highly aneuploid with considerable chromosomal variation, it is necessary to exclude the possibility that such shifts are due to the random selection of cell clones with different chromosome complement. A recent critical study of Vogt indicates that karyotype and phenotype may be directly related to each other in certain situations (*10*).

MATERIALS AND METHODS

The maintenance of mouse strains, the histology and transplantation behavior of the tumors used, and the methods of transplantation are described in another paper (*5*).

Tumors.—Three dependent interstitial-cell tumors were used for cytological studies, denoted TNSB, TNSC, and TNSE. They all originated in (A/Sn × A.SW)F_1 male mice, which, at the age of 1 month, received subcutaneously a pellet containing 1 mg of diethylstilbestrol + 4 mg cholesterol (*3*). They were tested by serial transplantation to pellet-bearing and to untreated isologous males and females and to mice belonging to the parental strains.

As a rule, the testicular tumors did not grow in untreated isologous males, but grew well in diethylstilbestrol-treated males and females and more slowly in untreated females. However, a few autonomous sublines capable of growth in untreated males could be isolated. Two such lines, TNSB-AUT I and TNSB-AUT II, were used in this study. The former line was isolated from group TNSB-Ur which received 12.5 mg of urethan weekly. The detailed history of these lines appears in another paper (*5*), showing that the main lines, TNSB and TNSB-Ur, from which the autonomous sublines were isolated, remained estrogen-dependent, and the occasional takes in untreated males occurred randomly and infrequently, usually only in 1 mouse of an inoculated group. As judged from the frequency of takes and growth rate, TNSB-AUT I grew similarly in conditioned mice as the dependent TNSB tumor. The difference in untreated males was striking (table 1). TNSB-AUT II grew in 3 of 3 untreated, isologous males.

TABLE 1.—Summary of transplantation tests with tumors used for karyological analyses

Tumor	$(A/Sn \times A.SW)F_1$ males	
	With diethylstilbestrol pellet*	Without diethylstilbestrol*
TNSB	21/28	1/27†
TNSB-Ur‡	8/10	2/14§
TNSB-AUT I‖	3/3	12/13
TNSB-AUT II¶		3/3
TNSC	19/19	1/23
TNSE	6/9	0/8

*Number of mice killed by tumors over total number inoculated.
†Donor of the TNSB-AUT II line.
‡Started from a tumor belonging to the third transfer of TNSB. Mice were given 12.5 mg urethan intraperitoneally weekly.
§Donor of the TNSB-AUT I line.
‖From the third transfer of TNSB-Ur and subsequently propagated in untreated males.
¶From the third transfer of TNSB and subsequently transferred to untreated males.

From the testicular tumors a few isoantigenic variant sublines have been isolated, selectively compatible with one or the other of the parental strains (*5*). One such variant subline, TNSB-A, isolated from the TNSB-Ur line in the parental strain A and still dependent on estrogenic stimulation was also studied cytologically.

All tumors used for transplantation were examined histologically to minimize the risks of contamination, *e.g.*, with mammary carcinomas. The different tumor lines showed a similar histological picture with sheets of large, pale, polygonal cells having round or oval nuclei, several nucleoli, and faintly stained chromatin (*5*). There was little polymorphism. Hemorrhagic areas were present in most samples, and there were groups of small, round cells (*5*) similar to those found by Gardner (*11*) and by Shimkin *et al.* (*12*), which probably represent myelopoietic cells.

Cytologic methods.—A slight modification of the colchicine-hypotonic citrate method of Ford and Hamerton (*13*), described earlier (*14*), was used.

Chromosomal counts were made on drawings prepared with an Abbe's drawing apparatus. Only cells with intact outlines and well-dispersed chromosomes were counted.

RESULTS

The chromosome counts are recorded in table 2. Representative metaphase plates are pictured in figures 1, 2, and 3.

All three dependent tumors, TNSB, TNSC, and TNSE, were heteroploid, and their stemline cells contained 53 (fig. 1), 47 (fig. 2), and 52 chromosomes (fig. 3), respectively. No typical markers could be identified. The most detailed analysis was made on TNSB and different variant sublines derived from it.

TABLE 2.—Summary of

Tumor				Tumor-bearing mouse			Number of chromosomes						
Number of sample	Name	Transfer generations	Ability to grow in untreated males	Sex and treatment*	Time after inoculation (weeks)	Number of cells analyzed	31	32	33	34	35	36	37
1	TNSB	1	—	♀−	9	50	1			2			
2		1	—	♀−	46	28							
3		3	—	♀+	25	50		1					
4		3	—	♀+	29	27							
5		3	—	♀−	25	27							
6		4	—	♂+	8	25							
7		4	—	♂+	9	31							
8		4	—	♀+	9	34							
9		4	—	♀+	12	34					1		
10		4	—	♀+	8	30							
11		4	—	♀−	31	24							
12		4	—	♀−	31	38						1	
13		6	—	♀−	10	33							
Total TNSB, dependent						431	1	1		2	1	1	
14	TNSB-AUT I	2†	+	♂+	7	39							
15		2†	+	♀+	8	23							
16		2†	+	♀−	8	19							
17		2†	+	♀−	13	27							
18		3†	+	♀+	8	23							
19		3†	+	♀+	8	30							
20		4†	+	♂−	8	34							
Total TNSB-AUT I, autonomous						195							
21	TNSB-AUT II	2‡	+	♂+	9	28							
22	TNSB-A	2§	—	♂+	18	20							
23		2§	—	♂+	8	20							
24		3§	—	♂+	6	30							
25		3§	—	♂+	6	26							
Total TNSB-A, dependent, isoantigenic variant compatible with strain A						96							
26	TNSC	4	—	♀−	17	32							
27		5	—	♀+	9	30							
28		5	—	♂+	11	27							
29		6	—	♀+	9	29							
Total TNSC, dependent						118							
30	TNSE	3	—	♂+	11	39							

*+ = Diethylstilbestrol pellet implanted before transplantation; − = no pellet.
†Preceded by 4 transfers as dependent tumor in isologous hosts.

Diploid cells with 40 chromosomes were frequent in several tumors, and in a few TNSB samples they were predominant (#3 and 12). It is possible that these diploid cells belonged to another histological type, since they were much smaller than the rest of the population and similar to the small, round cells seen in histological sections. These were presumably myelopoietic cells, as stated earlier.

The variation around the stemline chromosome numbers was greater in samples collected from the first-transfer generations of TNSB than on later passages. One TNSB tumor of the first passage appeared to have

chromosome counts

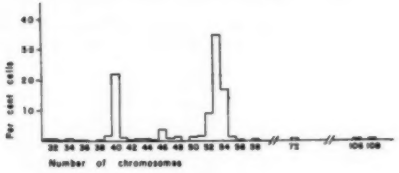
Number of Chromosomes—Continued																										
38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	72	80	86	92	94	106	108
		16					1	10	2	3			1	2	3	7	1	1									
		1	1	1	1	1				1		2	2	3	13	2											
2	1	20	1		1	1								3	13	5	2										
	1	3											1	1	11	9	1										
		1										1	1	5	18	1											
	2							2	1	1		1		4	10	2	1			1							
		3					1						1	1	12	13											
				2	1									9	15	7											
		3	1					1						2	19	6					1						
			1			1						1		4	3	17	1										2
		5												2	16	1‡											
	2	31													4												
	1	4												3	22	2										1	
2	7	87	4	3	3	3	2	13	3	5		5	6	39	159	72	6	1		1	1					1	2
		4						1	1	1			2	3	10	15	1					1					
			1		1					1		2	2	2	14												
						1				1			1	1	13	2											
		7										1		1	17	1											
		6												1	14	2											
		3												2	21	4											
		4											1	5	20	4											
		24	1		1	1		1	1	3		3	6	15	109	28	1					1					
		2										1	2	4	17	2											
		14												4	2												
		11											2	3	4												
								1				2	5	19	2	1											
					1					1	1	1	3	9	8	2											
		25			1			1		1	1	3	10	35	16	3											
		1		1				3	24	2															1		
			2	2	1	2	1	7	14														1				
		1						2	20	2														1	1		
			2			1		5	21																		
		2	4	3	1	3	1	17	79	4													1	1	2		
1		3	1										3	29	2												

‡Preceded by 2 transfers as dependent tumor in isologous hosts.
§Preceded by 7 transfers as dependent tumor in isologous hosts.

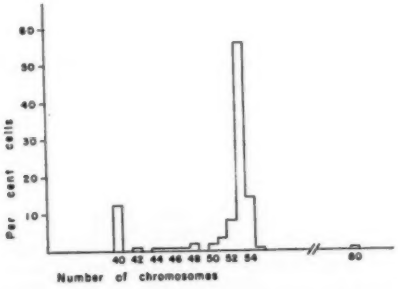
an additional stem line with 46 chromosomes, which later disappeared. A few hypodiploid cells with chromosome numbers 31 to 39 were found in dependent TNSB tumors. It cannot be excluded that they represent squashing artifacts.

The first autonomous subline studied, TNSB-AUT I, resembled the dependent form of TNSB cytologically (figs. 1 and 3). Its stem line contained 53 chromosomes, and no differences were detected in chromosomal morphology between TNSB and TNSB-AUT I. The extent of variation around the stemline number was similar to that of TNSB

(text-figs. 1 and 2). Chromosome numbers were slightly more variable in the second-transfer generation of TNSB-AUT I as compared to its third and fourth transfers or the later passages of TNSB.



TEXT-FIGURE 1.—Chromosome number distribution in TNSB; 431 cells analyzed.



TEXT-FIGURE 2.—Chromosome number distribution in TNSB-AUT I; 195 cells analyzed.

From the second autonomous subline, TNSB-AUT II, only one tumor was studied. It resembled TNSB-AUT I.

Cytological examination of TNSB-A, the estrogen-dependent isoantigenic variant of TNSB, compatible with the parental strain A, showed a stem line containing 52 chromosomes instead of 53 as in TNSB. In the sample examined from the second TNSB-A transfer most cells had 40 chromosomes, probably corresponding to the earlier described "myelopoietic" group. In the sample taken from the third transfer in which the stem line with 52 chromosomes dominated, this group of cells was nonexistent.

DISCUSSION

Interstitial-cell tumors of the testis are suitable tools for the study of the progression of hormone-dependent tumors to autonomy (*3*, *4*, *11*). Dependent and independent sublines, differing in their ability to grow in untreated isologous males, can easily be obtained and characterized. The material lends itself well for chromosome cytological studies.

The present work has shown that three different dependent tumors were characterized by heteroploid modal chromosome numbers, different for each tumor. No further chromosomal changes could be detected in

the transition of one dependent tumor line to autonomy, observed on two different occasions. In most samples there was also a minor stem line with 40 chromosomes, possibly corresponding to myelopoietic cells, which have been described by Gardner (*11*) and Shimkin *et al.* (*12*) to occur in primary and transplanted interstitial-cell tumors.

It is remarkable that the stem lines were heteroploid on the first transfer of TNSB, which showed that the cytogenetic change was not a late consequence of tumor progression. Unfortunately, it was not possible to make any chromosomal analyses on primary, induced interstitial-cell tumors. While transplantation itself may impose selection pressures favoring heteroploid cells (*9*), many primary tumors are clearly heteroploid (*7, 14, 15–18*), though pure diploid tumors undoubtedly exist (*14, 19–20*).

The fact that dependent tumors were heteroploid can be interpreted to mean that their cells are cytogenetically distinct from normal cells. Therefore, these results do not support the concept (*1*) that dependent tumors consist of essentially normal cells. The results are more in agreement with biological studies that indicate a difference between dependent tumors and normal hyperplastic cells. In thyrotropic pituitary tumors, Furth *et al.* observed a gradual transition to autonomy through different degrees of hormone dependence (*21*). Andervont *et al.* (*3*) observed clusters of morphologically changed interstitial cells 16 weeks after diethylstilbestrol was administered to mice, and these cells were present up to 15 months after the discontinuation of the treatment. Interstitial-cell tumors started to grow immediately when estrogen was administered, and sometimes also began to grow in the absence of estrogenic stimulation and formed autonomous tumors (*3, 4*).

The absence of detectable chromosomal changes in the transition to autonomy cannot be used to draw inferences as to the nature of this change and its genetic versus epigenetic nature (*22*), since point mutations or minor chromosomal rearrangements cannot be excluded.

One isoantigenic variant subline, TNSB-A, isolated from TNSB and selectively compatible with strain A, had stemline cells with 52 chromosomes instead of 53 as in TNSB. Ising found a decrease in modal chromosome number when mouse tumors were transplanted to hamsters (*23*). Bayreuther and Hellström (unpublished data) also found a decrease in modal chromosome number with the transition of amethopterin-sensitive mouse lymphoma cells to resistance. Differences in the chromosome complements were found by Bayreuther and Klein (*24*) between one heterozygous F_1 methylcholanthrene-induced fibrosarcoma and several parental strain variants isolated. However, no differences in chromosome numbers were found between F_1 hybrid lymphomas and their isoantigenic variants compatible with one of the parental strains (*25*). It cannot be decided whether the slight difference in stemline number between TNSB and its derivative TNSB-A is related to the changed phenotype of the variant cells, or is an expression of ordinary random variation.

The finding that all three tumors studied, TNSB, TNSC, and TNSE, were different in their stemline karyotype is another example of the individuality of tumors found previously in methylcholanthrene-induced sarcomas (*14*) and in lymphomas (*7*). A similar individuality exists also in the isoantigenic variation in sarcomas, carcinomas, and lymphomas (*26*) and in the antigenic specificities of methylcholanthrene-induced fibrosarcomas in autologous or isologous hosts (*27*).

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PLATES

PLATE 88

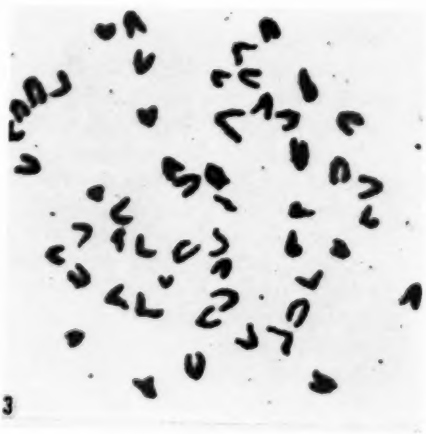
FIGURE 1.—TNSB metaphase (53 chromosomes). × 1,300

FIGURE 2.—TNSC metaphase (47 chromosomes). × 1,300

FIGURE 3.—TNSB–AUT I metaphase (53 chromosomes). × 1,300

1

2



3

Tumors of the Central Nervous System of Rats: With Report of Two Tumors of the Spinal Cord and Comments on Posterior Paralysis [1, 2]

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SUMMARY

The topic of tumors of the central nervous system of rats is reviewed. An ependymoma and a sarcoma of the spinal cord are reported. Comments are made on the clinical problem of posterior paralysis in rats and the difficulties in neurologic diagnosis in rodents.—J. Nat. Cancer Inst. 26: 719–735, 1961.

THE GENERAL topic of spontaneous tumors of the nervous system of laboratory animals was reviewed by Stochdorph (*1*) and specifically in rats by Snell *et al.* (*2*). Most of the references from the latter are mentioned here. There is a dearth of reports, and we know little about total incidence and types of neoplasms in the nervous system relative to involvement of other organ systems in rats and then related to strain and age of rats. Whether this concerns insufficient neuropathologic search or whether there is a truly low incidence in rats is a matter to ponder. Our inclination is to favor the former view. Gliomas, meningeal, hypophyseal, pineal, and plexus tumors have been regarded as extremely rare in domesticated and laboratory animals, but until thousands of animal brains are examined from all species the true position will remain uncertain. Only a small fraction of the total annual deaths of domesticated animals in any country is examined by a pathologist and in only a very few of these are the brain and spinal cord examined as a routine measure [*see* Innes and Saunders (*3*)]. Many tumors will certainly escape detection unless in an advanced stage. The statements perhaps are more appropriate for the small laboratory animals, rats and mice, in which clinical signs need never be dramatic or specific, and Peers (*4*) found cerebral tumors, experimentally produced, in mice that were clinically asymptomatic. For example, tumors of the nervous system in dogs have been found to be much more common than was generally believed—when meticulous attention is paid to clinical and pathologic study (*3*). We have found considerable histo-

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logic work necessary to provide a pathologic answer to the question of causation of neuroparalytic syndromes in rats, often loosely designated as posterior paralysis.

Rats are used extensively for diverse experimental neurologic studies, including research on the production of cerebral tumors by carcinogenic agents and irradiation [*see* Peers (*4*), Zimmerman (*5*), Russell (*6*), Weisburger and Weisburger (*7*), Symeonidis (*8*), and Snell *et al.*(*2*)]. We should thus be aware of the incidence and nature of neural tumors which occur spontaneously (*cf.* the incidence of spontaneous leukemia and mammary tumors in rats). This statement must not be taken as querulous doubt of the validity of the data of these workers that brain tumors cannot be produced by carcinogenic agents, or that their results could have been complicated by the presence of spontaneous tumors. Too many tumors of too great a variety of types have been produced in rats and mice (*4–6*) for any doubt to remain on that score.

The incidence of all tumors of the human nervous system lies between 2 to 5 percent of all human malignant disease (*9*). Peers (*10*) found that in 10,592 autopsies done in 39 years (1896 to 1936) in Boston and out of which some 5,150 heads had been examined, there were 902 intracranial tumors—*i.e.*, over 17 percent. We have a long way to go before a similar statement can be made about rats. There is no published study of a large number of rats with particular reference to the whole nervous system and to include a number of different strains of rats [*cf.* Bullock and Curtis (*11*), and Curtis *et al.* (*12*)]. The number of rats used to study senile changes in the nervous system are too small to have any significance on tumor incidence. Unfortunately, compared to a more positive approach to the experimental cancer problem, such a routine search for tumors in the brain and spinal cord of rats is tedious, costly, and by no means attractive to workers, or institutes, as a research project. The few papers dealing with spontaneous tumors of the nervous system of rats are as follows:

McCoy (*13*) in an examination of 100,000 *wild* rats in California—during a plague eradication campaign—found 103 tumors, and Woolley and Wherry (*14*) in a similar venture found, out of 23,000 rats, 23 with tumors; the brain was not mentioned. In such a study on wild rats, the important factor of age could not be assessed and with the numbers quoted, it would have been truly remarkable if the brain and spinal cord had been examined. The nervous system is not mentioned in the report by Bullock and Curtis (*11*) on the occurrence of 489 rats with tumors out of a total of 2,450, nor again by Curtis *et al.* (*12*) on autopsies on 31,868 rats (7 strains)—except that a ganglioneuroma of the optic nerve was found in one rat. Saxton *et al.* (*15*), in their nutritional studies related to tumors, mentioned the occurrence of one glioblastoma in an underfed rat [Osborne-Mendel (Yale) strain]. Their observations on pituitary tumors are dealt with later. Davis *et al.* (*16*) observed an incidence of 47 percent of mammary tumors in 150 Sprague-Dawley rats, but found no brain tumors. Ratcliffe (*17*), in his collection of 273 tumors in rats, which ac-

counted for 3 percent of all deaths, does not mention the nervous system. Highman and Altland (*18*) found one pontine glioma in one rat in a series of high-altitude exposure experiments. There are almost no references to neural tumors in rats by Stochdorph (*1*). It is relevant that there is a similar paucity of reported data in mice. Stewart *et al.* (*19*) described 2 identical tumors of the spinal nerve roots and cord that arose from the meninges or nerve roots; they were the first such neural tumors seen in more than 70,000 mice examined clinically and pathologically at the National Cancer Institute, Bethesda, Maryland. It would be important to know whether all of these mice had died natural deaths, and in how many of these was the nervous system examined. In view of the 2 spinal tumors in rats, 1 irradiated and 1 nonirradiated, it is also of interest that 1 mouse of Stewart *et al.* had been irradiated and the other had not. However, any work on the role of irradiation on the production of neural tumors should take into account natural incidence. Snell *et al.* (*2*) found malignant intracranial neurilemmoma and medulloblastoma in rats fed *N,N'*-fluoren-2,7-ylenebisacetamide. Dr. V. J. Rosen, U.S. Naval Radiological Defense Laboratory, San Francisco, California, has informed us that in examining 250 irradiated and nonirradiated Sprague-Dawley rats, he has found 3 intracranial tumors, 2 glioma and 1 meningioma, and 2 neurilemmoma of the trigeminal nerves.

The position regarding pituitary tumors in rats is different. It is beyond dispute that pituitary tumors, mostly chromophobe adenomas, have a high incidence in aging rats, but vary according to the strain. Saxton *et al.* (*15*)—*see* also Saxton and Graham (*20*)—reported 92 pituitary tumors, 69 in control rats and 33 in those subjected to a nutritional experiment. Gilbert *et al.* (*21*) analyzed material on 55 such tumors and Crain (*22*) on 11. Wolfe *et al.* (*23*) found that 68 percent of aged Wistar strain females had pituitary tumors. That the incidence in old rats is influenced by a number of different factors is evident from the work of Saxton *et al.* (*15*) on the nutritional status and by Kullander on the effects of estrogens and irradiation. Kullander (*24*) found that the youngest rat with a pituitary tumor was 12 months old, that growths were equally common in males and females, and that no influence on incidence was exerted by gonadectomy of males or females, but that incidence was promoted by total-body irradiation in intact and spayed female rats.

We have also seen chromophobe adenoma of the pituitary in adult rats, irradiated and nonirradiated. The animals showed a similar clinical picture to those with tumors of posterior paralysis, with, in addition, some circling and head tilting. (*See* our later comments on clinical signs.) The hypophyseal tumors were massive, around 5 to 7 × 3 to 4 mm in longest diameters.

TUMORS

"Ependymoma," Spinal Cord, Rat

The rat was about 1 year old and untreated. It started to show hind-leg weakness which progressed until it was totally incapacitated poste-

riorly, but it still was able to move with difficulty by pulling itself along by the forelegs (*cf.* fig. 6). The tail was flaccid. The rat was killed and the whole nervous system, intact in its bony covering, was fixed for examination. When the vertebral column was sliced after decalcification, there was an obvious grayish color of the dorsal white columns, as opposed to a normal pearly white, in the lower cervical/thoracic cord. The brain and spinal cord were examined by a series of slices cut at every 1 to 2 mm (about 28 blocks from spinal column alone). The tumor was plotted regarding its distribution (fig. 1, A to G). It could then be visualized that the tumor had grown longitudinally in the cord, and had not extended beyond the leptomeninx or caused any great distortion of the cord and canal. It must have been long and spindle-shaped with narrowing growing ends. It started about cervical 2 to 3 and extended well into the upper thoracic level. From the illustrations (figs. 2 and 3) the tumor in its widest diameter had involved almost two thirds of the cord (gray and white matter). A narrow rim of normal white matter was left mainly around the ventro-lateral columns, and there was evidence of secondary degeneration. The latter extended up and down in the ventral columns of white matter and to the lowest levels of the cord.

The tumor was composed of closely compact cords and diffuse masses of round to spindle-shaped cells (figs. 4 and 5) with hypochromatic nuclei and with little intercellular stroma (after Wilder's reticulum silver impregnation method). The reticulin fibers were all related to small vessels. The tumor stood out as a pale island in Luxol-blue preparations. There was no particular pattern, or any arrangement in whorls, rosettes, pseudorosettes, or cavities. In general, the structure was that of a highly cellular astrocytoma; the fibrillary components were brought out by special glial methods. At the caudal end of the tumor the relationship to the subendymal glia seemed well defined and there were tumor cells in the central canal. There were also discrete and separate subarachnoid infiltrations and implantations of tumor cells around nerve roots, all some distance down from the actual tumor, also illustrated in the 2 mice of Stewart *et al.* (*19*). It would seem to be the first recorded in the spinal cord of rats, and in this connection it is of interest to recall that ependymoma in man accounts for some 60 percent of spinal tumors.

Those who correlate clinical neurologic signs of animals with neuropathologic changes may find it of interest that the pyramidal tract is the site of this tumor in the spinal cord. In the rat, however, all the fibers of the latter decussate in the medulla and cross into the dorsal white columns. The tract then fades rapidly by the thoracic level. Another interesting feature of the pyramidal tract of the rat is that it is made up largely of nonmedullated fibers.

Polymorphic Cell Sarcoma or Malignant Meningioma, Spinal Cord, Irradiated Rat

The rat was one of a group of animals (Long-Evans rats) exposed to 400 r (total body) when it was 40 days old. It became paralyzed (fig. 6)

in the hindlegs and showed incontinence 366 days later which increased in severity. It was killed after 21 days' duration of its neuroparalytic signs. The brain and spinal cord were examined as before.

Macroscopically a tumor was identified which involved the lumbar enlargement of the spinal cord and *cauda equina*. There was very slight involvement of the lumbar nerves above the level of the tumor. The latter started about lumbar 3 and gradually with swelling obliterated the whole structure of the cord. At its widest diameter, about 2 mm, it had caused, by pressure, enlargement of the vertebral canal (fig. 7). The lumbar nerves below the tumor showed secondary degeneration. The tumor started to fade by the end of the lumbar level, but tumor cells were still to be found around the *filum terminale*. The tumor at one part invaded the intervertebral foramina and had involved and destroyed most of the spinal (lumbar) nerves, but there was no destruction of the bony covering, though there was some evidence of stimulated, periosteal, osteoblastic activity. The surrounding vertebral muscles at all levels were normal. The clinical signs in this animal appeared to be clearly explained.

The tumor was a highly cellular one (figs. 8, 9, and 10) having mostly spindle-shaped or fusiform cells with large nuclei (fig. 9), occasionally with some palisading, and rarely with any whorling. In parts the cells formed a mosaic of closely packed, large, round, and polygonal cells. To a large extent the tumor growth had remained confined within the *dura mater*, but had pushed it out and caused distention of the canal. There were no psammoma bodies, and the tumor was unlike the classical meningioma. There was abundant intercellular fibrillary reticulum (after Wilder's method), which had a distinct streaming vertical to the surface of the tumor, or reticular fibers radiated out with the perithelial arrangements of tumor cells around vessels (fig. 10).

Some meningiomas have been figured both in human and animal neuropathology which have a resemblance to this tumor. The problem of classification of so-called malignant meningioma and sarcomas arising from the meninges has been discussed by Russell and Rubenstein (*25*). There is no clear evidence that this rat tumor arose from the meninges, and from the series of sections examined it seems certain that it originated in the cord itself.

Innes and Saunders (*3*) have commented on the inherent difficulty of classifying many neural tumors of animals according to current patterns in human neuropathology. The possibility that the tumor was secondary was considered and excluded. The rat had been exposed to whole-body irradiation in a study on production of tumors of mammary and thyroid glands. No malignant tumor was present in those situations. A lymphoma was present in one lumbar lymph node, obviously different in form to that described and illustrated. No comment can be made on the possible role of the irradiation in producing the tumor, but it is incorrect to state, as is still done, that the nervous system is relatively insensitive to X radiation.

COMMENTS

Neurologic examination of rats, or any small laboratory animal, can be of only a very limited and objective nature and is little guide to what may be found on examination of the brain and spinal cord. Times without number, it will be found that there is little of specific value in clinical neurologic signs, merely because of the relatively simple functional specialization of the small rodent brain. Lesions may be clinically silent, and with given neuroparalytic types it may take laborious histologic work to establish any definitive pathologic diagnosis.

In the course of experimental work (*26*) on postirradiation myelitis leading to malacia of the spinal cord of rats, pituitary tumors and 2 spinal-cord cases have been found because of the extent of the method of examination adopted for the irradiation work. Essentially, all animals with tumors and irradiation lesions of the spinal cord showed a basic common clinical pattern, except for the head tilting and circling in the pituitary tumor cases. There was incontinence of urine, motor weakness of the hindlegs that increased in severity to incoordination and ataxia, and finally complete loss of control over posterior mobility and bladder and rectal sphincters. There was no flaccid paralysis of the hindlegs even at the end, but the tail had remarkably little tonus and was almost flaccid. Such animals could move around by pulling themselves by their forelegs with the hindlegs dragging behind.

An indistinguishable neuroparalytic syndrome has now been reported in the following conditions of rats, listed by Innes and Saunders (*3*) all of which are totally different pathologically: 1) spontaneous demyelinating disease of adult rats; 2) prolonged vitamin E deficiency in *adult* rats in which there are lesions with a deposit of a fluorescent, acid-fast material in degenerative changes in the spinal cord, apart from the well-known skeletal myodegeneration; 3) the myelitis and malacia of the spinal cord produced, after a long latent, clinically silent, period, by localized radiation of the vertebral column; 4) the reported present cases of spinal tumors, one in the cervical thoracic level and one at lumbar to *cauda equina* level; 5) pressure effects of pituitary tumors. To this list can be appended the cases of Highman and Altland (*18*), who observed, in addition to a pontine glioma in one rat, rats with partial paralysis of the hindlegs and other signs, and which on examination revealed hemorrhage in the subarachnoid space, ventricles, and central canal in 3 rats, and extradural hemorrhage of the spinal cord in one. "Wobbly" rats have also been reported as a possible inherited condition—with no neuropathologic basis ever defined (*27*). This merely pinpoints the difficulties of neurologic diagnosis in rats, and that, unless sufficient attention is paid to study of the brain and cord, no satisfactory explanation for the signs will be obtained. This is important to those who use rats for experimental purposes, particularly when effects on the nervous system are studied, and to those in charge of rat colonies who study the pathology of natural deaths.

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PLATES

580132—61——21

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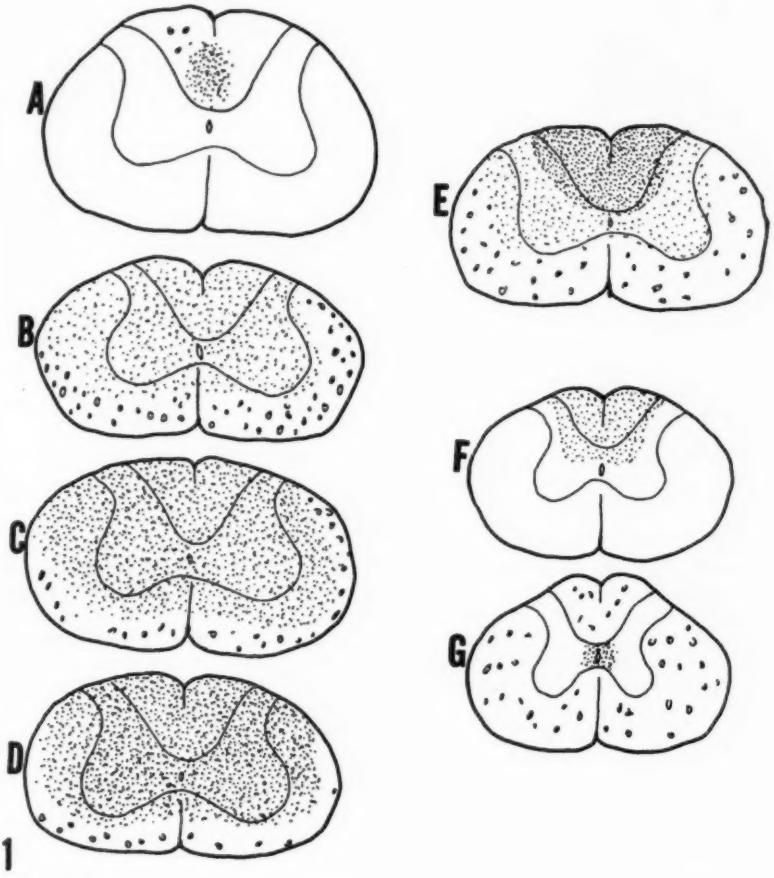
PLATE 89

FIGURE 1.—"Ependymoma," rat. Spinal cord. Diagram of topographic spread of tumor within the cord, slices of the latter being cut about 1 mm thick (A through G); starting at A which was cervical 3, tumor involved last part of cervical cord and beginning of thoracic. Shaded parts represent tumor. Small circles—secondary degeneration. *Cf.* figures 2 and 3.


A
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G
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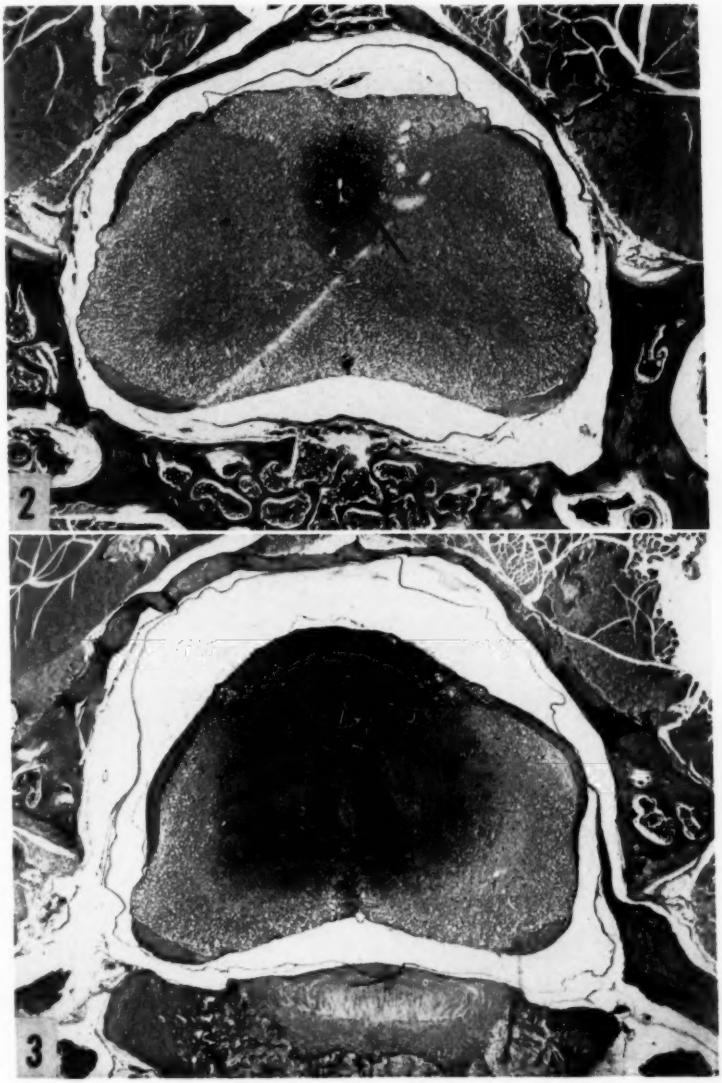
PLATE 90

FIGURES 2 AND 3.—Same case as figure 1. Figure 2: low power showing the most cranial level of tumor, involving very small area above central canal in dorsal columns of white matter only. Hematoxylin and eosin. × 16. Figure 3: tumor at its widest diameter, corresponding to about figure 1C. All parts of dorsal columns of white matter and total gray matter involved; a marginated area of normal white matter in ventro-lateral columns remains, but even at this power shows a porosity due to secondary degeneration. Hematoxylin and eosin. About × 16

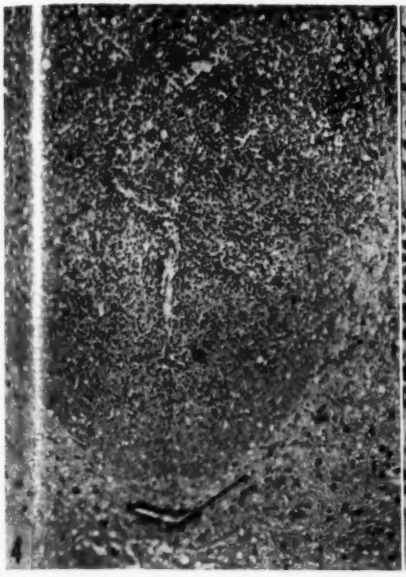


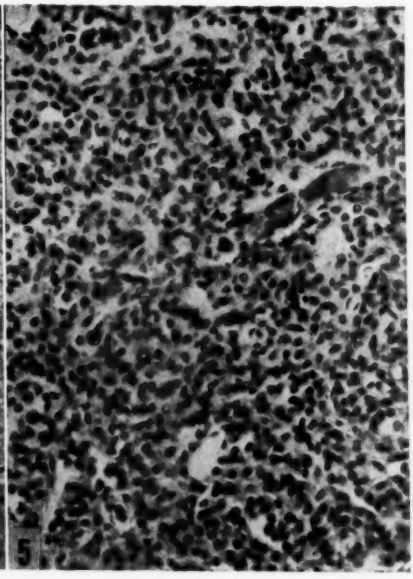
580132—61——22

PLATE 91

FIGURES 4 AND 5.—Same case as figures 1, 2, and 3. Figure 4: area above central canal taken from about the same zone depicted in figure 2. Involvement of dorsal white column. × 80. Figure 5: the uniform appearance of fusiform and round cells without whorls or rosettes. Hematoxylin and eosin. × 200

FIGURE 6.—Sarcoma, spinal cord, rat. Appearance of rat before death. Animal with posterior paralysis caught in act of pulling itself along with forelegs.





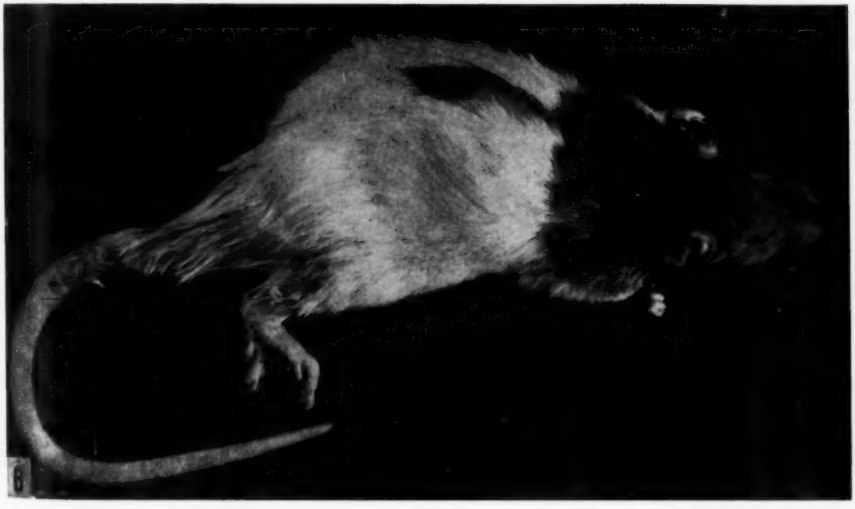


PLATE 92

FIGURE 7.—Same case as figure 6. Topographic spread of tumor. Diagram
slices of spinal cord cut about 1 mm thick (A through E). Starting at A, which
about lumbar 3; tumor spread down to termination of cord and *cauda equina* (
and E).

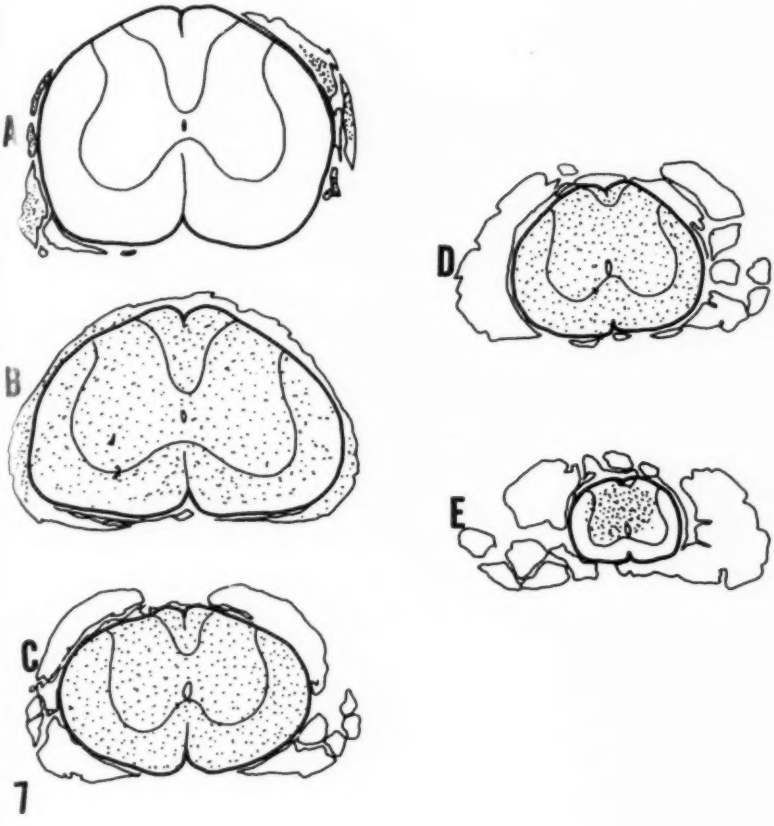

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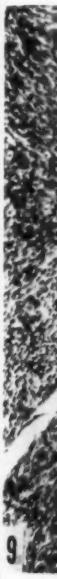
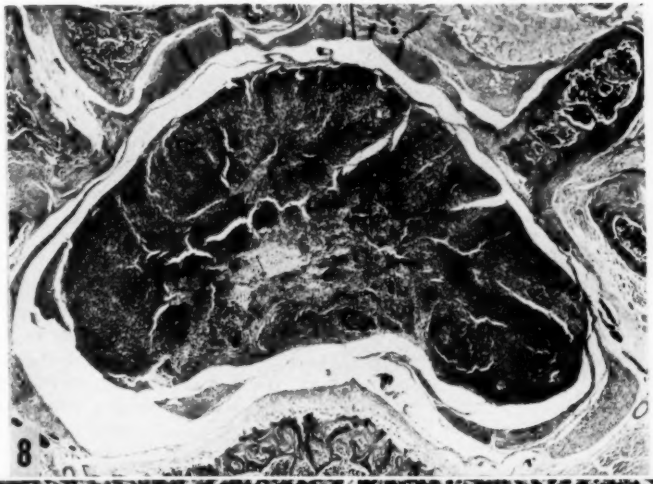
PLATE 93

FIGURES 8, 9, AND 10.—Same case as figures 6 and 7. Figure 8: massive involvement of whole of cord in lumbar enlargement. × 10. Figure 9: spindle-shaped cells without whorls. Hematoxylin and eosin. × 200. Figure 10: showing reticulum fibrils radiating out from a vessel. Wilder's reticulum method. × 200

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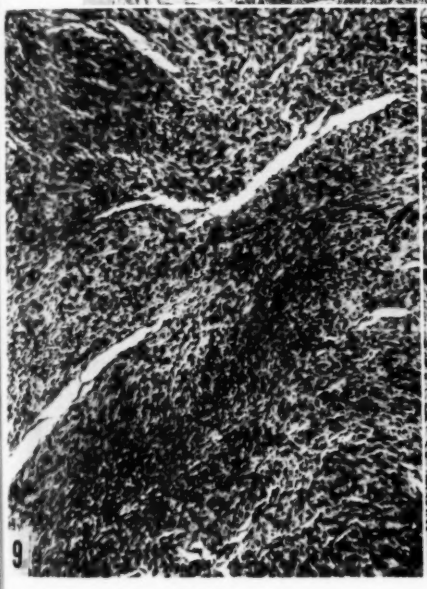
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Tumor Induction by Genetically Homogeneous Lines of Polyoma Virus [1, 2]

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SUMMARY

Polyoma virus, reisolated from mouse parotid, kidney, and mammary-tumor cells in culture, has been purified by 3 successive single plaque isolations. Inoculation of the third-passage single plaque isolates into hamsters, mice, and rats can result in the development of multiple tumors. Homogeneous lines of polyoma virus are therefore capable of inducing more than one tumor type. Plaque-purified virus of kidney and mammary-tumor origin induced parotid tumors in mice to the same degree as virus initially isolated from the parotid-tumor cells. However, virus of kidney-tumor origin appeared to have a greater ability for producing multiple tumors in AKR mice than virus of parotid-tumor origin. Data have been obtained which suggest that the dosage, in addition to its effect on the proportion of animals developing tumors, also appears to determine how many of these will develop multiple tumors after inoculation with plaque-purified virus. The results have also provided further evidence that there is a difference in susceptibility of different strains of mice. With the use of the same plaque-purified virus at the same dosage level, AKR mice had a much higher susceptibility than SWR mice, but C57BL/6 mice in these experiments were completely resistant to tumor induction. Thus, despite the fact that the same plaque-purified virus can produce tumors in different rodents, there still appears to be some strain specificity in mice.—J. Nat. Cancer Inst. 26: 737-753, 1961.

THE POLYOMA virus can induce a wide variety of tumors in different species (*1–5*). It was therefore of interest to determine whether the multiplicity of tumor types results from a mixture of different virus strains, or whether a single virus type can induce different types of tumors. A similar question has been raised concerning chicken-tumor viruses (*6, 7*).

The application of bacteriophage plaque techniques to animal viruses (*8*) provides a method for the production of genetically homogeneous lines of virus and an experimental approach to the above problem. Preliminary studies showed that single plaque isolates can produce multiple tumors in mice and hamsters (*9*). It has also been noted (*10*) that virus

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[2] Aided by research grant C-5266 from the National Cancer Institute, National Institutes of Health, Public Health Service.

stocks raised from 6 single plaque isolates produced no difference in the types of tumors induced in mice. The present investigation deals with tumor induction in different strains of mice, in hamsters, and in rats by genetically homogeneous virus populations originally reisolated from 3 polyoma-induced mouse tumors. The homogeneity of these virus populations has been ensured by the use of 3 successive plaque isolations. In addition, the response of hamsters to inoculation with a polyoma plaque-type mutant, isolated from polyoma-infected Earle's strain L cells, has been examined.

MATERIALS AND METHODS

Virus.—The 3 sources of polyoma virus used in this study, which have been designated IL-11, IL-5, and IL-12 (*9*), were originally reisolated by harvesting the growth medium of trypsin-dispersed mouse parotid, mammary, and kidney-tumor cells in culture (*11–13*). These tumors were induced in mice by polyoma virus, line 3919 (*1*), received through the courtesy of Dr. S. E. Stewart and Dr. B. E. Eddy.

Virus titration.—The plaque assay procedures used for virus titration were as previously described (*9*, *14*).

Production of plaques for single plaque isolation.—Mouse-embryo primary tissue cultures were inoculated with serial dilutions (twofold around the endpoint) of virus calculated to produce a small number of plaques per plate. After 3 hours for virus adsorption, the plates were washed twice with LA medium (Earle's saline with 0.5% lactalbumin hydrolysate) plus 10 percent horse serum to remove unadsorbed inoculum. The plates were then overlaid with an agar-nutrient mixture, incubated at 37° C, and refed on day 6 of incubation with a second agar-nutrient overlay containing neutral red, as previously described (*14*). On days 10 to 12 of incubation, the plates were examined and those which contained 1 to 5 well-separated plaques were selected for plaque isolation.

Plaque isolation.—The position of the plaque to be isolated was marked on the underside of the plate. A 2 ml pipette was then inserted through the agar-overlay so that its tip made contact with the marked plaque, and a cylinder of the agar-overlay directly above the plaque was withdrawn into the pipette. This was then suspended in 1 ml of LA medium plus 2 percent horse serum. Titration of many plaque isolates obtained in this way showed an average virus content of 3.6×10^4 plaque-forming units (PFU).

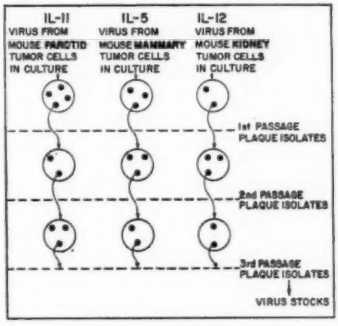
Plaque-purification procedure.—To improve the homogeneity of the virus population isolated from a single plaque, the plaque isolates were serially passaged 3 times. That is, virus obtained from a previous plaque isolate was suitably diluted, inoculated onto mouse-embryo tissue-culture monolayers, and 1 of the resulting plaques picked and suspended in 1 ml of medium as described. Where required, third-passage plaque isolates were made into high-titer virus stocks as previously described (*14*).

Animals.—Mice were of the inbred lines AKR, SWR, and C57BL/6; the breeding animals were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. Hamsters and rats were from random-bred colonies. Tests for polyoma virus contamination of the breeding room in this laboratory have all been negative.

Inoculations.—Mice were inoculated subcutaneously 1 to 3 days after birth with 0.05 ml of virus; hamsters were inoculated subcutaneously 2 to 5 days after birth with 0.1 ml; and rats were inoculated intracerebrally (*5*) 2 to 3 days after birth with 0.05 ml.

EXPERIMENTS AND RESULTS

An outline of the experimental procedure used is given in text-figure 1. Each of the 3 sources of virus (IL-5, IL-11, and IL-12) was purified by 3 successive plaque isolations, as described under "Methods," directly after isolation from the tumor cells. Single plaque isolates from each of the three passages were then inoculated into mice, hamsters, and rats (at least 1 litter per isolate). Third-passage single plaque isolates were also diluted 1:1 and each then served as inoculum for several litters of mice and hamsters. To test the response of mice and rats to high-titer, plaque-purified virus, stocks titering 10^7 to 10^8 PFU per ml were made from the third-passage single plaque isolates.


IL-11
VIRUS FROM MOUSE PAROTID TUMOR CELLS IN CULTURE
IL-5
VIRUS FROM MOUSE MAMMARY TUMOR CELLS IN CULTURE
IL-12
VIRUS FROM MOUSE KIDNEY TUMOR CELLS IN CULTURE
1st PASSAGE PLAQUE ISOLATES
2nd PASSAGE PLAQUE ISOLATES
3rd PASSAGE PLAQUE ISOLATES
VIRUS STOCKS

TEXT-FIGURE 1.—Outline of procedure used for production of homogeneous lines of polyoma virus derived from 3 sources.

After inoculation, the animals were observed for 6 to 12 months or until death. They were killed only when it appeared likely that they would die at a time when autopsy could not be performed. At autopsy, the animals were examined for gross tumors and the organs fixed for histological examination. Animals were scored as having single or multiple tumors. The single tumors in mice were predominantly parotid tumors (both unilateral and bilateral), those in hamsters were subcutaneous sarcomas, while those in rats were either kidney or skull tumors. Mice

were scored as having multiple tumors if they had parotid plus mammary, and/or hair follicle, and/or kidney tumors. Hamsters were scored as having multiple tumors if they had subcutaneous sarcomas plus heart sarcomas (in most cases) and/or kidney and/or lung tumors. Multiple tumors in rats consisted of kidney plus skull tumors. All gross diagnoses were confirmed by histological examination.

Virus Content of Single Plaque Isolates

Since it was not feasible to determine the virus content of each of the 59 plaque isolates inoculated, 16 were chosen at random and titrated by plaque assay. The results are shown in table 1. The variation in virus content between the majority of plaque isolates is not large. Calculation of the virus input per animal in the succeeding experiments was thus based upon an average value of 3.6×10^4 PFU per plaque isolate suspended in 1 ml of medium. Where high-titer virus stocks were inoculated, the virus input per animal was determined from a direct titration of the stock.

TABLE 1.—Virus content of single plaque isolates

Plaque isolate No.	Virus content (PFU)
1	1.2×10^4
2	0.8×10^4
3	12.0×10^4
4	3.6×10^4
5	1.2×10^4
6	5.2×10^4
7	1.6×10^4
8	3.6×10^4
9	2.4×10^4
10	5.6×10^4
11	1.2×10^4
12	2.4×10^4
13	2.0×10^4
14	2.4×10^4
15	8.0×10^4
16	5.0×10^4
Mean:	3.6×10^4

Response of Hamsters, Mice, and Rats to Inoculation With Plaque-Purified Virus

The response of hamsters to inoculation with the 3 virus sources, purified by 1, 2, and 3 successive plaque isolations, is shown in table 2. These hamsters were inoculated directly with single plaque isolates (1.8 and 3.6×10^3 PFU per animal). Virus purified 3 times was capable of producing multiple tumors in 12 of 35, or 34 percent, of the hamsters with tumors. Irrespective of the source of the virus (whether of mouse kidney, mammary, or parotid-tumor origin) the single tumors were subcutaneous sarcomas, and the multiple tumors were subcutaneous

VOL. 26, NO. 3, MARCH 1961
580132—61——23

TABLE 2.—Tumor induction in hamsters inoculated with single plaque isolates

Inoculum*										
Line of virus	Number of serial plaque purifications	Number of single plaque isolates inoculated	Number of litters inoculated	Number of hamsters inoculated	Number of hamsters surviving more than 1 month	Number of hamsters with single tumors†	Number of hamsters with multiple tumors	Number of hamsters without tumors	Died unclassified‡	Observation period (months)
IL-12, kidney	1	1	1	7	6	2	2	0	2	6
	2	1	1	6	6	4	0	1	1	9
	3	4	5	31	24	8	7	3	6	8
IL-5, mammary	1	6	6	49	35	14	14	1	6	4
	2	2	2	12	10	5	4	0	1	4
	3	2	3	22	19	8	1	4	6	7
IL-11, parotid	2	1	1	7	7	1	4	0	2	5
	3	4	6	45	28	7	4	6	11	8
Totals: 3 lines combined	1	7	7	56	41	16	16	1	8	
	2	4	4	25	23	10	8	1	4	
	3	10	14	98	71	23	12	13	23	

*PFU inoculated per hamster in different experiments, 1.8 to 3.6 $\times 10^3$.
†Single tumors were subcutaneous sarcomas. Multiple tumors were subcutaneous sarcomas plus heart sarcomas and/or kidney and/or lung tumors.
‡Autopsy could not be performed because of cannibalism or other causes.

sarcomas plus heart and/or kidney and/or lung tumors. The data on the hamsters also suggest that virus from the third-passage isolates may have become attenuated as to its tumor-producing ability, since the number of inoculated hamsters remaining free of tumors is larger in this group than in the groups inoculated with those of first- and second-passage plaque isolates. However, in view of the low virus input per hamster and the absence of fuller dosage-response data, these results do not seem to warrant a definite conclusion in this respect.

Tumor induction in AKR mice inoculated with plaque-purified virus both at low (plaque isolates) and high (stock from plaque isolates) dosage levels is shown in table 3. It will be noted that 3-times plaque-purified virus was capable of inducing multiple tumors in 15 of 45 (33%) mice with tumors, and that the proportion of multiple tumors in mice inoculated with the plaque-purified virus of kidney-tumor origin was greater at high dosage levels, 1.5×10^7 PFU per mouse, than at lower inputs, 1.8×10^3 PFU per mouse. Plaque-purified virus of the kidney or mammary sources had the same probability of initiating a parotid tumor as virus initially isolated from the parotid-tumor cells. However, the virus of kidney-tumor origin produced a higher frequency of multiple tumors than the virus of parotid-tumor origin. This is particularly illustrated by the observation that multiple tumors appeared in 2 of 8 mice inoculated with 1.8×10^3 PFU of IL-12 virus, whereas none appeared in 6 mice inoculated with 1.5×10^6 PFU of IL-11 virus. The very low proportion of AKR mice remaining free of tumors testifies to the high susceptibility of this strain to tumor induction with polyoma virus.

Data on SWR mice inoculated with single plaque isolates are shown in table 4. It will be noted first that SWR mice are less susceptible than AKR to tumor induction with polyoma virus. For 9 to 11 months after inoculation, 101 of 207, or 48 percent, remained free of tumors. As with AKR mice, plaque-purified virus from the kidney and mammary-tumor sources had the same probability of initiating a parotid tumor as virus originally isolated from parotid-tumor cells. The proportion of SWR mice developing multiple tumors after inoculation with plaque-purified virus was 6 of 44, or 14 percent, of the mice with tumors.

In table 5, the response of AKR and SWR mice and hamsters to inoculation with the *same* third-passage plaque isolate is shown. In this experiment, the third-passage plaque isolates were diluted 1:1 to have inoculum sufficient for several litters. Although the virus input per animal was low, multiple tumors did appear in AKR mice and hamsters. The data also show clearly the higher susceptibility of AKR mice and hamsters compared to SWR mice inoculated with the same low dosage of plaque-purified virus. As in the previous cases presented, virus from plaque isolates of kidney and mammary-tumor origin induced parotid tumors in mice and subcutaneous sarcomas in hamsters, to the same degree as virus from plaque isolates of parotid-tumor origin. It is interesting also to note that here again multiple tumors were most frequent with IL-12 virus, and there were none with IL-11 virus.

TABLE 3.—Tumor induction in AKR mice inoculated with 3-times plaque-purified virus

Line of virus	Number of third-passage single plaque isolates inoculated	Number of litters inoculated	Number of mice inoculated	PFU inoculated per mouse	Number of mice surviving more than 1 month	Number of mice with single tumors*	Number of mice with multiple tumors	Number of mice without tumors	Died unclassified	Observation period (months)
IL-12, kidney	2	3	19	1.8×10^3	15	6	2	3	4	8
	—†	3	15	1.5×10^7	14	1	12	0	1	6
IL-5, mammary	4	4	24	1.8×10^3	22	14	1	0	7	9
IL-11, parotid	1	1	6	1.8×10^3	4	3	0	0	1	8
	—†	2	8	1.5×10^6	7	6	0	0	1	8
Totals:		13	72		62	30	15	3	14	

* Single tumors were parotid tumors. Multiple tumors were parotid plus mammary and/or hair follicle and/or kidney tumors.
† Virus stocks made from third-passage single plaque isolates.

TABLE 4.—Tumor induction in SWR mice inoculated with single plaque isolates

Inoculum*										
Line of virus	Number of serial plaque purifications	Number of single plaque isolates inoculated	Number of litters inoculated	Number of mice inoculated	Number of mice surviving more than 1 month	Number of mice with single tumors	Number of mice with multiple tumors	Number of mice without tumors	Died unclassified	Observation period (months)
IL-12, kidney	1	2	4	26	16	1	0	12	3	10
	3	5	8	51	43	8	2	33	0	9
IL-5, mammary	1	2	2	16	13	4	0	3	6	11
	2	3	5	30	26	8	1	10	7	10
	3	5	10	62	47	14	2	23	8	9
IL-11, parotid	1	2	5	31	25	9	0	8	8	9
	3	5	8	56	37	16	2	12	7	9
Totals: 3 lines combined	1	6	11	73	54	14	0	23	17	
	2	3	5	30	26	8	1	10	7	
	3	15	26	169	127	38	6	68	15	

*PFU inoculated per mouse in different experiments, 0.9 to 1.8×10^3.

TABLE 5.—Third-passage single plaque isolates inoculated into mice and hamsters

Third-passage plaque isolate No.*	Origin of isolate	Inoculated into 1 litter of:	Number of animals inoculated	Number surviving more than 1 month	Number with single tumor	Number with multiple tumors	Number without tumors†	Died unclassified
71-8-D	IL-12, kidney	SWR	9	9	3	0	6	0
		Hamster	5	3	0	1	0	2
71-8-E	IL-12, kidney	AKR	8	6	2	1	0	3
		Hamster	7	5	3	1	1	0
71-10-A	IL-12, kidney	SWR	6	6	1	0	5	0
		SWR	4	4	2	0	2	0
		AKR	6	5	2	0	3	0
		AKR	5	4	2	1	0	1
		Hamster	7	6	2	1	2	1
71-2-B	IL-5, mammary	AKR	5	4	4	0	0	0
		Hamster	5	3	1	1	0	1
		Hamster	9	9	4	0	1	4
71-2-C	IL-5, mammary	SWR	7	6	1	0	4	1
		SWR	4	4	1	0	3	0
		AKR	6	5	4	0	0	1
		Hamster	8	7	3	0	3	1
71-18-E	IL-11, parotid	SWR	7	7	1	0	4	2
		AKR	7	6	6	0	0	0
		Hamster	7	3	2	0	1	0
71-18-I	IL-11, parotid	SWR	7	6	1	0	5	0
		SWR	8	7	2	0	4	1
		Hamster	8	4	3	0	1	0

*In this experiment, each plaque isolate which was originally suspended in 1 ml of medium was further diluted 1:1 to have sufficient inoculum. Mice were inoculated with 0.9×10^3 PFU each; hamsters with 1.8×10^3 PFU each.

†The observation period was 8 months.

The composite data, combined from lines IL-5, IL-11, and IL-12, of tumor induction in mice, hamsters, and rats inoculated with 3-times plaque-purified virus at both high and low dosage levels are shown in table 6. The following conclusions can be drawn from this table: 1) In all animals susceptible to tumor induction with polyoma virus, *i.e.*, excluding the C57BL/6 mice used in these experiments, 3-times plaque-purified virus was capable of initiating the development of multiple tumors, 35 of 134, or 26 percent, of the animals with tumors; 2) hamsters and AKR mice are much more susceptible to tumor induction than SWR mice or rats; and 3) C57BL/6 mice appear to have been resistant to tumor induction. A relative lack of tumor induction in C57BL mice has also been observed by others (*15*). Furthermore, the results of the AKR mice inoculated with 3-times plaque-purified virus at high and low dosage levels show that the proportion of mice with multiple tumors was significantly greater at high input levels than at low input levels. Thus the dosage-response factor, in addition to affecting the proportion of animals developing tumors, also appears to determine what proportion of these will develop multiple tumors even after inoculation with plaque-purified virus.

Tumor Induction in Hamsters Inoculated With a Polyoma Virus Mutant Released by Earle's Strain L Cells

In the course of experiments on growing wild-type polyoma virus on Earle's strain L cells, a small plaque mutant form of the virus was isolated. Cultures of strain L cells were inoculated with wild-type polyoma virus (5×10^5 PFU onto 1×10^6 cells), washed after adsorption, and then maintained in LA medium plus 20 percent horse serum with medium change every 3 to 4 days. Subcultures of the inoculated strain L cells were made at 4, 14, 25, 31, and 38 days after inoculation. During this time there was no evidence of any cytopathic effect. However, 44 days after the original inoculation, *i.e.*, during the fifth subculture, signs of degeneration began to appear, and, in contrast to the control series, all further attempts to subculture these cells failed.

Harvests of the supernatant fluids of the inoculated strain L cell cultures at 26, 44, 58, and 73 days post inoculation, *i.e.*, from the third subculture onward, were titrated by plaque assay on mouse-embryo monolayers and found to contain 4.8×10^3, 5.0×10^5, 5.0×10^5, and 1×10^6 PFU/ml, respectively, of a small plaque polyoma virus mutant designated as sp2. The size and appearance of this sp2 plaque mutant were similar to those reported for another sp mutant of polyoma (sp1), isolated from a polyoma-infected line of mouse-embryo cells (*16*). In cross-neutralization tests with anti-wild-type polyoma antiserum, the sp2 mutant (like sp1) was fully neutralized. Examination of approximately 5,000 plaques produced by sp2 isolated direct from strain L cells revealed no wild-type large plaques. With reference to the plaque size marker, sp2 virus isolated from strain L cells thus appears to be homogeneous. Furthermore, two

TABLE 6.—Composite table of tumor induction in mice, hamsters, and rats with 3-times plaque-purified virus*

Animal	Dosage†	Number of litters inoculated	Number of animals inoculated	Number surviving more than 1 month	Number with single tumors‡	Number with multiple tumors	Number without tumors	Died unclassified §	Average observation period (months)
Hamster	Low	14	98	71	23	12	13	23	6.4
AKR	Low	8	49	41	23	3	3	12	8.3
AKR	High	5	23	21	7	12	0	2	7.5
SWR	Low	26	169	127	38	6	68	15	9.5
C57BL/6	High	2	14	12	0	0	12	0	11.0
Rats	Low	6	52	33	8	1	19	5	9.0
Rats	High	1	4	1	0	1	0	0	8.0
Totals:		62	409	306	99	35	115	57	

*Data from lines IL-5, IL-11, and IL-12 are combined in this table.
†High = 1.5 to 10.0 × 10^8 PFU inoculated per animal in different experiments.
Low = 0.9 to 3.6 × 10^8 PFU inoculated per animal in different experiments.
‡For description of single and multiple tumors, *see* text.
§Autopsy could not be performed because of cannibalism or other causes

passages of sp2 on mouse-embryo cells produced no change in the type of small plaque developing on mouse-embryo assay plates.

As previously reported (*17*), the sp1 mutant isolated directly from the line of mouse-embryo cells showed some attenuation in its tumor-inducing properties. A test of the tumor-inducing ability of sp2 (isolated direct from strain L cells) was therefore carried out. Eighteen hamsters (3 litters) were inoculated each with 5.0×10^4 to 1.0×10^5 PFU of sp2. Nine hamsters survived more than 1 month, and of these 6 developed subcutaneous sarcomas during an observation period of 5 months. With respect to tumor induction in hamsters, therefore, these data do not suggest any strong degree of attenuation of the sp2 mutant.

DISCUSSION

It has been shown in the present experiments that multiple tumors can develop in hamsters, mice, and rats inoculated with third-passage single plaque isolates. It is therefore concluded that homogeneous lines of polyoma virus are capable of initiating the development of multiple tumors. Furthermore, it has been found that third-passage single plaque isolates of mammary and kidney-tumor origin have the same probability of initiating a parotid tumor in mice as plaque isolates of parotid-tumor origin. However, some of the data have indicated that the homogeneous line originally reisolated from the kidney-tumor cells had a greater ability for inducing multiple tumors than the line derived from the parotid-tumor cells. This and previous data on the sp1 mutant (*17*) indicate that the tumor-inducing capacity of polyoma virus can become attenuated.

The technique used for the production of genetically homogeneous lines of polyoma virus was identical with that for the production of such lines in other animal viruses (*8*). The homogeneity of the virus population of the final single plaque isolates inoculated into animals was thus ensured by 1) the selection of plaques from plates inoculated at limiting dilutions (1 to 5 well-separated plaques per plate), and 2) the 3 successive passages of the plaque isolates. The validity of the application of the plaque-purification technique to polyoma virus has recently been questioned (*18*) in view of the fact that this virus is capable of infecting mouse cells *in vitro* in both a lytic (resulting in cell death) and nonlytic fashion (*14*, *16*, *18–21*). It was therefore suggested (*18*) that the viral population isolated from a single plaque (approximately 10^4 PFU) may be contaminated with some virus released from adjacent infected, but viable, cells which had undergone a nonlytic interaction. The possibility of this type of contamination appears to us to be remote in view of the previously mentioned methods for ensuring homogeneity. In addition, recent work (*13*) has indicated that if progeny virus is produced after the nonlytic interaction, it is produced by means of a lytic cycle of multiplication.

The direct inoculation of single plaque isolates has shown that at this low dosage (approximately 10^3 PFU per animal) some of the inoculated

animals remained free of tumors 9 to 12 months after inoculation. It has also been shown that AKR mice inoculated at higher dosage levels (10^6 to 10^7 PFU per mouse) show a higher proportion of multiple tumors than AKR mice inoculated at the lower level of 10^3 PFU per mouse. Since it thus appears likely that not every cell-virus meeting will result in the induction of a tumor, the higher proportion of multiple tumors at higher dosage levels may be explained on the basis of a greater number of cell-virus impacts, in the same animal, destined to produce tumors.

The results have also shown a difference in susceptibility of different strains of mice to tumor induction with the same plaque-purified virus at the same dosage level. AKR mice showed a greater susceptibility to tumor induction than SWR, and no tumors were observed in C57BL/6 mice. A high susceptibility of AKR mice and a relatively low susceptibility of the C57BR and C57BL strains have also been reported by others (*7*, *22–24*). Despite the fact that polyoma virus can produce tumors in different rodents, there is still some strain specificity in mice.

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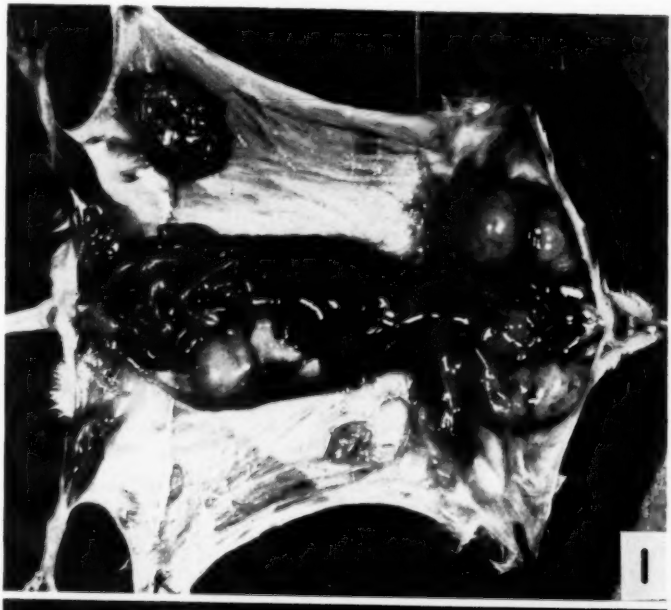
PLATES

PLATE 94

FIGURE 1.— ♂ AKR mouse with parotid, mammary, kidney, and hair follicle tumors, 3½ months after subcutaneous inoculation with stock virus made from a third-passage single plaque isolate (II-12).

FIGURE 2.—Plaques on mouse-embryo monolayers, 15 days after inoculation with polyoma virus. Plaques of this type and distribution were chosen for single plaque isolations.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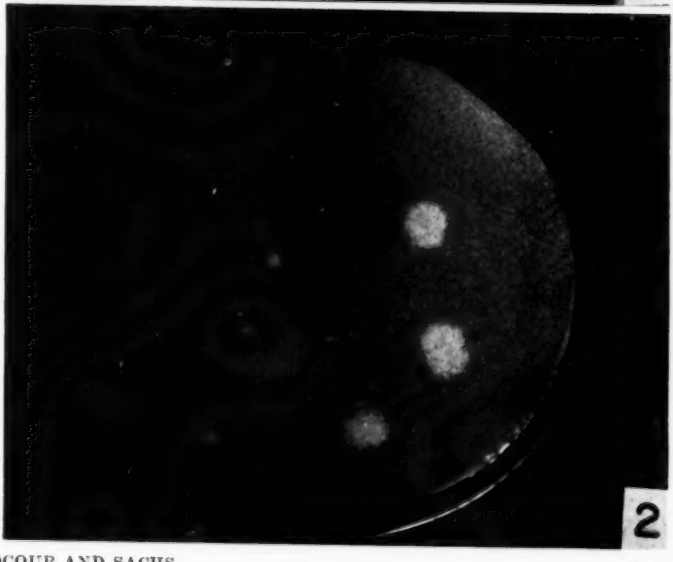
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PLATE 95

FIGURE 3.—♂ AKR mouse with parotid and mammary tumors, 4½ months after subcutaneous inoculation with third-passage single plaque isolate (IL-12).

FIGURE 4.—Hamster showing subcutaneous sarcomas, 3 months after subcutaneous inoculation with third-passage single plaque isolate (IL-12).

3

4

Rabbit Heart Cell Culture, Strain RHF-1. I. Growth, Cytology, Histology, and Susceptibility to Some Inclusion-Inducing Viruses[1,2]

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SUMMARY

A strain of fibroblast-like cells, rabbit heart cell culture, strain RHF-1, derived from the heart tissue of a normal domestic rabbit, has been cultivated serially *in vitro* over a period of 2 years. Morphologically, this strain has maintained its characteristic fibroblast-like appearance throughout the period *in vitro*. Chromosomal observation at intervals during serial transfers has shown conformity of type of chromosomes with those of the domestic rabbit while some variation is evidenced in numbers of each type correspondent to the degree of ploidy of that cell. Evidence is also presented that herpes simplex, pseudorabies, and vaccinia viruses grow and induce specific lesions in the cell cultures of strain RHF-1.—J. Nat. Cancer Inst. 26: 755–773, 1961.

IT HAS been frequently shown that cell cultures derived from various sources differ in their susceptibility to viral agents. There is still considerable need for permanent strains of specific cell types for the study of virus-host cell relationship. It is the purpose of this report to describe (*a*) the successful establishment of a strain of fibroblasts derived from normal domestic rabbit heart tissue, strain RHF-1, as well as its chromosomal complement through several generations, and (*b*) the propagation of herpes simplex, pseudorabies, and vaccinia viruses in the cultures of strain RHF-1 with the production of consistent and typical histologic changes.

MATERIALS AND METHODS

Rabbit heart cell culture.—The heart was removed aseptically from 6- to 7-week-old normal domestic rabbits and the muscle was minced in medium consisting of mixture 199 plus 2 percent calf serum. The fragments were washed 3 times with the medium and once with warm trypsin

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(0.25%) in Earle's balanced salt solution at *p*H 7.4. The washed tissue was dispersed with trypsin for a total of 2 hours at room temperature; three steps of washings with the medium followed. The cells were resuspended in growth medium, consisting of mixture 199, 10 percent calf serum, 0.1 percent lactalbumin hydrolysate, and adjusted to *p*H 7.3 to 7.4 with $NaHCO_3$ (2.8%). Penicillin (100 units/ml), streptomycin (100 μg/ml), and Mycostatin (100 units/ml) were also added. The cells in concentration of approximately 1.5×10^5/ml were planted in serum bottles and the cultures were then incubated at 37° C. Growth was usually well developed after 6 to 7 days of incubation, at which time the monolayers were dispersed with trypsin, and subcultures were prepared by reseeding new bottles with the desired inoculum of cells. During the first ten serial passages, it was necessary to supplement the growth medium with 50 percent of "conditioned" medium (medium obtained from preceding passages that had supported the growth of cells). In this manner a continuous serial culture of rabbit heart cells has been successfully established in tissue culture. This line has been designated as RHF-1 to indicate the animal and tissue from which the culture was originally derived and the histology of the cell type.

Cell count.—Cells from stock cultures were dispersed by addition of trypsin followed by incubation for 5 minutes at 37° C. The pooled suspension of cells was lightly centrifuged for 10 minutes and the cells were then resuspended in known volume of fresh medium, counted in a hemocytometer, and then inoculated into a series of new bottles. At 24-hour intervals during the following 9 days the cell contents of 4 bottles were treated with trypsin, pooled, and resuspended in their original volume with fresh medium. The average number of live cells per ml was determined according to the method of Kanda and Melnick (*1*).

Cytology.—Preparations for chromosome study were made from cells grown on coverglasses in short-necked Leighton tubes or from suspensions of trypsinized cells grown as a monolayer in bottles. The cells were treated with colchicine (2.5 μg per ml) for 3 hours at 37° C and were subjected to a 20-minute treatment with hypotonic medium prior to fixation (3:1 alcohol-acetic acid) and acetocarmine or aceto-orcein staining.

RESULTS

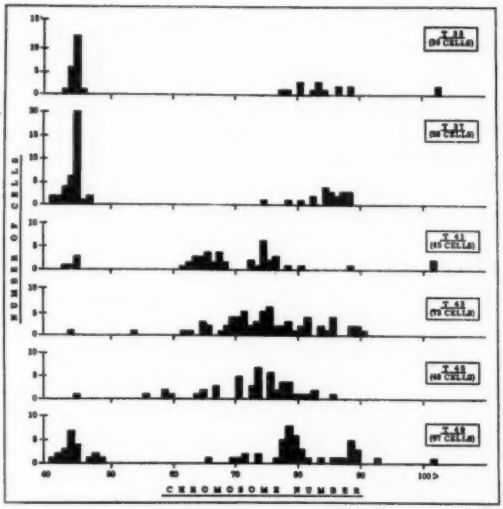
Cytologic Observations

Cytologic studies in RHF-1 cells were initiated at the 35th serial transfer (T35) and the material was sampled sporadically thereafter merely as a check on the possibility of contamination by other cell strains. Two sublines of RHF-1 were maintained in later subcultures. One was subcultured every 5 to 7 days, the other every 7 to 9 days. The distribution of chromosome numbers in the transfer generations is shown in text-figure 1. It is not implied, however, that this represents a sequential picture, since one or the other subline was chosen at random. In each case there is a

diploid component close to the normal rabbit chromosome number (44) and a polyploid component of the population. In the earlier generations (T35 to T37) this is close to the $4n$ number, but in later generations more hypotetraploidy appears. In the T65 (not shown in text-fig. 1) only 26 cells were counted. Of these, 6 were $2n$, 8 were $3n$, and 12 were tetraploid and hypotetraploid.

The chromosomes of the rabbit described by Melander (*2*) include 18 chromosomes with median centromeres, 14 to 17 chromosomes with submedian centromeres, and 9 to 11 acrocentric chromosomes.

We have observed the chromosomes of domestic white female rabbits in the primary and the first subculture of the heart cells. Our observations indicate that there are 16 to 17 chromosomes with median centromeres



TEXT-FIGURE 1.—Frequency distribution of chromosome numbers in RHF-1 cells in 6 different transfer generations of 2 subculture lines (*see* text).

(arm ratio$<$2:1), 15 to 16 chromosomes with submedian centromeres (arm ratio $>$2:1), and 12 acrocentric chromosomes (fig. 1). This is in close agreement with Melander's "idiogram analyses." Such an analysis of a cell from a primary culture of rabbit heart cell cultures is shown in figure 5. The chromosomes are ranked according to length and grouped according to centromere position. Representative cells from T35, T49, and T65 cultures have been selected to indicate the changes in chromosome morphology that have appeared. Figure 2 shows a cell from T35 culture with its karyotype in figure 6. There are 14 chromosomes with median centromeres, 17 with submedian centromeres, and 12 acrocentrics. In one chromosome the position of the centromere could not be ascertained. After the 35th serial passage of RHF-1 there was little change either in chromosome number or in their morphology.

The T49 population consisted mainly of hypotetraploid cells. Figure 3 shows a typical hypotetraploid cell with 76 chromosomes, and figure 7 is a karyotype of this cell. This cell has 21 chromosomes with median centromeres, 27 with submedian centromeres, and 24 acrocentrics. In the remaining 6 the centromere position could not be determined.

In both diploid and tetraploid components of the T65 population a new chromosomal type was observed to be predominant. The predominant cells are characterized by a diminuation in the number of acrocentrics and the appearance of an exceptionally long submetacentric (figs. 4 and 8). This chromosome appeared singly in the diploid cells and one or two such long chromosomes were also present in the polyploid cells. The changes that have been observed in the position of the centromere in RHF-1 cells are summarized in table 1. This table is based on measurements of the lengths of the long and short arms of each chromosome. It compares the rabbit chromosome morphology of lung squashes (*2*) or primary and first subcultures of rabbit heart cells with various cell generations of RHF-1 strain. In each cell the 3 classes of chromosomes are represented. The numbers approximate those found in the domestic rabbit heart cells. When changes in ploidy have occurred, the number in each group is also increased. The number of M, S, and A chromosomes is not exactly doubled, however, since the cell is heteroploid.

Table 1.—Chromosome morphology of domestic rabbit cells; position of centromere

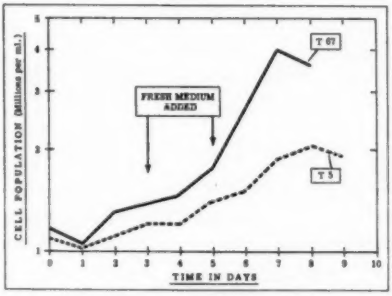
	Arm ratio <2:1 median (m) chromosomes	Arm ratio >2:1 submedian (s) chromosomes	Acrocentrics	Questionable	Total chromosomes
Normal domestic rabbit					
Rabbit heart					
Primary culture	17	15	12	—	44
T1 rabbit heart	16	16	12	—	44
Range	16–17	15–16	12		44
Embryonic lung cells (*2*)	18	14–17	9–11		44
RHF-1 cells					
T35	14	17	12	1	44
T49 cell 1	21	27	24	6	78
2	25	26	20	11	82
T65 cell 1	20	15	7	2	44
2	19	17	8	—	44
3	30	27	19	2	78

Histologic Appearance

During the first 20 subcultures strain RHF-1 was composed mainly of a heterogenous population of cells. Spindle-shaped cells, as well as polygonal and rounded cells with distinct nuclei, were the predominant cell types (fig. 9). As transfers progressed, however, the morphologic appearance of the strain became more uniform and the cellular sheet seemed to consist largely of fibroblast-like cells (fig. 10).

Growth Rates of Cells

Growth of strain RHF-1 cells was relatively poor in the absence of conditioned medium during the earlier passages, and the cultures failed to proliferate for more than a few serial subcultures. However, proliferation continued in a normal manner when conditioned medium was completely replaced by fresh medium after the tenth serial passage. As seen in text-figure 2, the cell growth progressed at a slow rate and was stimulated by renewal of medium 3 and 5 days after the seeding. The curves show that growth of the fifth serial transfer was much slower than the 67th cell passage. Under these conditions the time required for doubling in cell number was 7 to 8 days for the fifth passage cells and 5 to 6 days for the 67th passage cultures. As transfers passed the tenth passage level, though transient fluctuations in growth rate were frequently encountered, in general the rate of proliferation was more consistent throughout the subsequent generations.



TEXT-FIGURE 2.—Growth of rabbit heart cells in monolayer cultures. Each point in curves represents average values for 4 bottles. T5 and T67 refer to fifth and 67th serial transfers, respectively.

Herpes Simplex Virus

The morphologic changes induced by herpes simplex virus in the epithelial cells of rabbits' cornea cultivated *in vitro* (*3*), in normal rabbit and guinea-pig testes maintained in suspended cell cultures (*4*), in Earle's strain L fibroblasts (*5*), in chick fibroblasts (*6*), in HeLa (*7*), and in rabbit kidney cell cultures (*8*) have been well described. In the present experiments, lesions were first seen in cultures of RHF-1 cells at 12 to 14 hours after the inoculation of herpes virus ($10^{4.2}$ TCD50[5]). The earliest change detectable in the virus-infected cells consisted of fragmentation of chromatin followed by formation of nuclear inclusions usually separated from the nuclear membrane by a clear zone (fig. 11). At 24 hours post inoculation most of the cells contained well-developed inclusions. These inclusions were considered to represent a specific manifestation of herpes infections, since their specificity was readily ascertained by neutralization test with the use of herpes immune serum. In addition, the continued

[5] TCD50 = 50 percent tissue-culture dose.

replication of virus in RHF-1 cells could readily be detected by titration of each passage material in both RHF-1 and HeLa cell strains as shown in table 2. It can be seen that herpes virus readily multiplied and increased in titer through serial passages in RHF-1 cells with a cumulative dilution of the original material of 10^{-17}.

TABLE 2.—Serial propagation of herpes simplex virus in continuous cell culture derived from normal domestic rabbit heart, RHF-1

Cell passage No.	Virus passage No.	Total days in culture	Cumulative dilution of original inoculum (log10)	Cytopathogenic titer (log TCD50/ml)* for	
				RHF-1	HeLa
	Inoculum				6.5
38	1	2	0.6	6.3	5.5
40	2	4	1.2	6.5	5.5
40	3	5	1.8	6.5	5.3
42	4	9	2.3	6.3	5.3
42	5	14	5.9	6.6	5.5
45	6	20	11.0	7.0	5.3
45	7	25	17.0	7.0	5.6

*Five culture tubes each of RHF-1 and HeLa cells were used for each of tenfold dilutions. All tubes were incubated stationary at 36° C. The endpoint readings were terminated 7 days after the inoculation of cell cultures, and the 50 percent cytopathogenic titers were calculated by the method of Reed and Muench (9).

Pseudorabies Virus

The transmission of pseudorabies virus (Pr), kindly supplied by A. S. Kaplan, through 7 serial passages in RHF-1 cells was successfully demonstrated as shown in table 3. The presence of virus was detected by subinoculation of RHF-1, monkey kidney, and secondary rabbit kidney cell cultures with the fluid phases of each passage virus. It is apparent from the results of this experiment that strain RHF-1 was capable of supporting the growth of Pr virus and that the original virus after a $10^{-24.5}$ dilution contained more infective particles than the first passage inoculum. Furthermore, the identity of the virus from the first, fourth, and seventh passage series was reaffirmed with known Pr immune serum.

TABLE 3.—Serial propagation of pseudorabies virus in continuous cell culture derived from normal domestic rabbit heart, RHF-1

Cell passage No.	Virus passage No.	Total days in culture	Cumulative dilution of original inoculum (log10)	Cytopathogenic titer (log TCID50*/ml) for		
				RHF-1	MK†	RK‡
	Inoculum					6.2
37	1	1	1.1	5.0	3.5	3.5
38	2	2	1.8	7.3	3.5	
38	3	4	5.8	7.0	6.5	6.0
43	4	8	12.8	7.5	5.8	6.5
44	6	12	21.0	6.6	6.5	5.5
46	7	16	24.5	7.0	6.0	5.0

*TCID50 = 50 percent tissue-culture infectious dose.
†Monkey kidney culture.
‡Secondary culture of rabbit kidney cells.

The cytopathologic changes induced by Pr virus in RHF-1 cells were not unlike the changes described for Pr-rabbit kidney system (*8*, *10*). Inoculation of RHF-1 with Pr virus (10^3TCD50) resulted in the production of cellular lesions as early as 8 hours post inoculation, at which time there appeared to be a gradual enclosure of nuclei in the syncytium (fig. 12). By 12 to 16 hours post inoculation, large multinucleated syncytical masses joined by threadlike processes containing nuclear inclusions were predominantly present (fig. 13). Characteristic Pr inclusions have also been described in Earle's strain L cells, HeLa cells (*5*, *7*), and in monkey kidney cells (*11*).

Vaccinia Virus

Evidence for the multiplication of vaccinia virus in strain RHF-1 was obtained after inoculation of the monkey kidney-passaged virus that was kindly supplied by Dr. J. Warren. As can be seen in table 4, the virus readily multiplied throughout the passage series, and there was a gradual increase in virus titer despite the fact that total dilution of the original material was $10^{-12.5}$. Furthermore, infective material obtained from the final passage contained virus which was immunologically identical with the original inoculum. Cellular changes induced by vaccinia virus were slow at the beginning, but on subsequent subculturing the virus continued to multiply rapidly and to produce cytoplasmic inclusions that were generally surrounded by a clear zone (fig. 14). As the infection progressed, the number of cells with cytoplasmic inclusions also increased steadily until most of the cells in the culture contained one or more inclusions. Similar cytoplasmic changes have been described for vaccinia virus in various cell cultures (*3*, *7*, *12–14*).

TABLE 4.—Serial propagation of vaccinia virus in continuous cell culture derived from normal domestic rabbit heart, RHF-1

Cell passage No.	Virus passage No.	Total days in culture	Cumulative dilution of original inoculum (log 10)	Cytopathogenic titer (log TCID50/ml) for	
				RHF-1	MK*
	Inoculum				7.0
38	1	3	0.6	3.6	5.5
40	2	8	1.2	4.5	7.0
40	3	11	1.8	5.3	7.0
42	5	17	6.5	6.0	6.2
44	6	24	10.0	5.7	6.5
45	7	32	12.5	6.2	6.0

*Monkey kidney cells.

DISCUSSION

A strain of fibroblast-like cells derived from the heart tissue of a 6- to 7-week-old normal domestic rabbit has been investigated. Strain RHF-1 has been successfully cultivated serially *in vitro* over a 2-year period.

The chromosome complement of the rabbit has been described from squashes of embryonic rabbit lungs (*2*). The difficulty of Melander in identifying individual chromosomes was also encountered in our primary rabbit heart cell cultures. When the chromosomes were grouped according to centromere position, our observations and those of Melander were in good agreement (*see* table 1).

Changes in the chromosome numbers that are observed during long-term cultivation of cell lines have recently been discussed by Hsu and Klatt (*15*) for Novikoff rat hepatoma *in vitro*, and by Ruddle, Berman, and Stulberg (*16*) for cell lines derived from normal human peripheral blood. They observed diploid and polyploid modes in early generations superseded by true polyploidy, and, finally, as a result of nondisjunction or other mitotic anomalies, the appearance of hypotetraploids.

That the RHF-1 line has undergone this type of sequence may be observed in text-figure 1 where earlier generations are predominantly diploid and later generations heteroploid. The RHF-1 cells are quite similar in chromosome morphology to that of domestic rabbit primary cultures throughout the serial subcultivation. The only exception is the long submetacentric observed in the cells of T65. This chromosome could have originated from a translocation of a long submetacentric and another chromosome with at least one long arm, possibly an acrocentric, since they are diminished in number in this generation. Such translocations have been discussed by Levan and Biesele (*17*).

Comparison of the karyotypes of any cell generation of RHF-1 line with that of a primary rabbit culture or with Melander's karyotypes shows great similarity in the kinds of chromosomes represented in RHF-1 and the chromosomes of the domestic rabbit. The numbers within each group (S, M, A) vary with the total chromosome number of the cell. For this reason, it is believed that the strain of RHF-1 is purely of rabbit origin and has remained free of contamination with other cell lines during the period of subcultivation.

Evidence is presented that viruses of herpes simplex, pseudorabies, and vaccinia are capable of serial propagation in strain RHF-1. Furthermore, nuclear inclusion formation by herpes simplex and pseudorabies viruses and cytoplasmic inclusions induced by vaccinia virus in this strain were found to be specific, since their formation was readily prevented by the specific immune serum. These inclusions were at no time found in uninfected cultures.

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580132—61——26

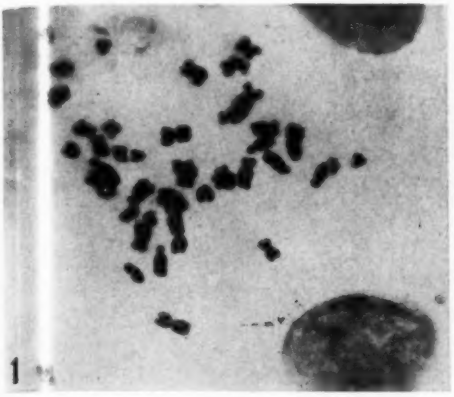
PLATE 96

FIGURE 1.—Chromosomes of domestic female rabbit in primary culture—44 chromosomes. × 1,500

FIGURE 2.—Chromosomes of RHF-1 (T35)—44 chromosomes. × 1,500

FIGURE 3.—Chromosomes of RHF-1 (T49)—78 chromosomes. × 1,500

FIGURE 4.—Chromosomes of RHF-1 (T65)—44 chromosomes. × 1,500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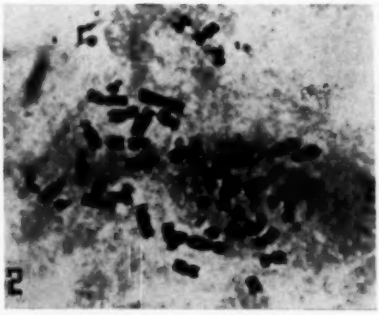
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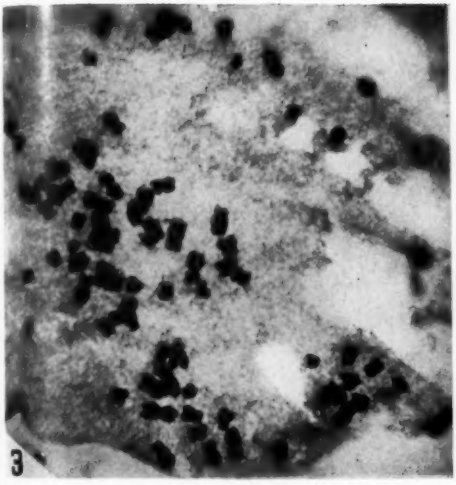
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PLATE 97

FIGURE 5.—Karyotype of chromosomes of domestic rabbit primary culture. Same as figure 1.

FIGURE 6.—Karyotype of chromosomes of RHF-1(T35). Same as figure 2.

T0
5
T35
6

PLATE 98

FIGURE 7.—Karyotype of chromosomes of RHF-1 (T49). Same as figure 3. Six chromosomes grouped to *left* on *lower* line were overlapped; length or centromere position could not be definitely determined.

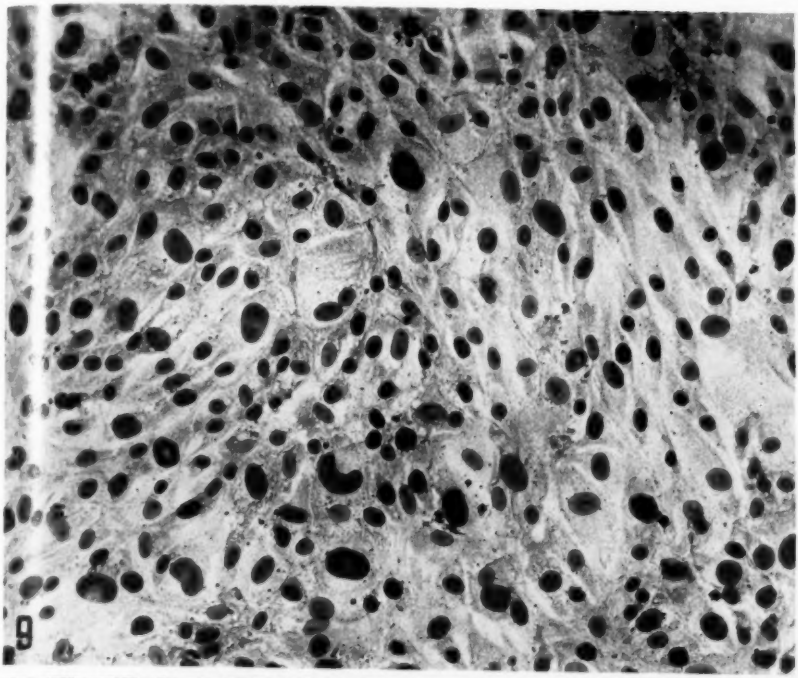
FIGURE 8.—Karyotype of RHF-1 cells (T65). Same as figure 4.

T49
7
T65
8

PLATE 99

FIGURE 9.—Cells from nineteenth passage (T19) level of strain RHF-1. *Note* heterogeneity of population; nuclei vary in size and shape. All histologic preparations were fixed in 95 percent ethyl alcohol and stained with hematoxylin and eosin. × 150

FIGURE 10.—Thirty-eight (T38) passage level of strain RHF-1. Cell population is uniformly composed of fibroblast-like cells. × 150



KHOOBYARIAN AND PALMER

580132—61——27

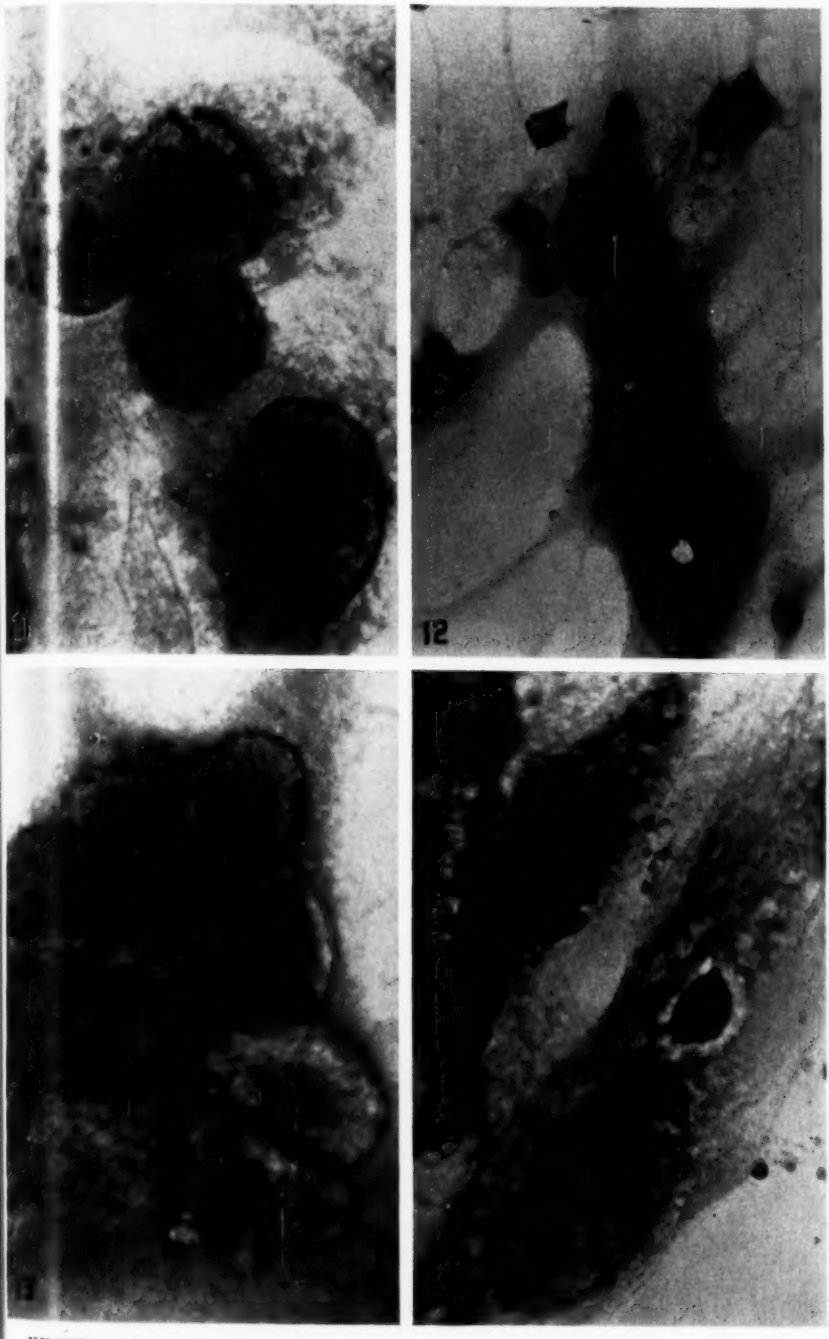
PLATE 100

FIGURE 11.—Cells of strain RHF-1 infected with herpes simplex virus show intranuclear inclusions surrounded by clear zone. Oil immersion. × 1,000

FIGURE 12.—Pseudorabies virus-induced syncytium in RHF-1 cells, 8 hours after inoculation. × 300

FIGURE 13.—Intranuclear inclusions of pseudorabies virus in cells of RHF-1 were generally formed at 12 to 16 hours post inoculation. Oil immersion. × 1,000

FIGURE 14.—Cells of strain RHF-1 24 hours after infection with vaccinia virus. *Note* cytoplasmic inclusion surrounded by a clear zone. Oil immersion. × 1,000


12

Dissemination of Rous Sarcoma Virus as a Cause of "Metastases" [1]

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SUMMARY

Chicks were given an inoculation of a cell-free preparation of Rous sarcoma virus into the wing web. After periods from 8 hours to 11 days, the inoculated wings were amputated under local anesthesia. Controls received the same virus inoculation but either were not amputated or had the uninoculated wing amputated. In the wing webs, sarcoma cells were first identified on day 4. On day 3 scattered cells of uncertain identity were sometimes found, but at 48 hours and earlier there was no evidence of tumor. Virus was not detectable by infectivity tests or by fluorescein-labeled antibody technique until day 4. The birds were killed at 15 or 25 days after inoculation so that the lungs could be studied for presence of tumors and virus, or were observed for 90 days to determine survival. Amputation of the virus-inoculated wing prior to day 5 delayed and decreased mortality and reduced the frequency with which sarcomas and Rous virus were found in lungs. However, even when amputation was performed 8, 16, 24, or 48 hours after inoculation, tumors and virus sometimes appeared in the lungs and some birds died of pulmonary sarcomas. Since amputation preceded appearance of sarcoma cells, these lung tumors must have resulted from early dissemination of virus from the inoculated wing.—J. Nat. Cancer Inst. 26: 775-783, 1961.

THE PRESENCE of visceral sarcoma in chicks bearing intramuscular Rous sarcoma was reported by Rous in 1910 (*1*). Several other reports since then have confirmed the spread of sarcoma to visceral organs. Whether these "metastases" are due to cellular emboli or to virus dissemination has not been resolved. This study was inspired by the observations in our laboratory that visceral lesions are often as large as the tumors at the site of inoculation and may occur in the occasional bird that does not develop a tumor at the site of inoculation.

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[2] The authors are indebted to Dr. W. Ray Bryan of the National Cancer Institute for supplying virus preparation, to Dr. Robert C. Mellors of The Hospital for Special Surgery for doing the fluorescein-labeled antibody studies, to Mr. Thomas Cardoza of this laboratory for technical assistance, and to the Phoebe Waterman Foundation for financial support.

580132—61——28

METHOD

White Leghorn chicks, 3 to 5 days of age (Shamrock Farms, North Brunswick, N.J.), were inoculated through the "forearm" muscles into the intracutaneous space of the wing web with 0.2 ml of a 10^{-2} dilution of a partially purified Rous sarcoma virus preparation supplied by Dr. Ray Bryan (preparation CT 694). This inoculum contained approximately 10,000 minimal tumor-forming doses, as measured by the same technique. The chicks were labeled with aluminum clips in the uninoculated wing and placed in groups of 15 with the help of random tables. At 8 hours, 16 hours, daily from day 1 to day 7, and on days 9 and 11 after inoculation, the inoculated wings of birds in these groups were amputated at the shoulder joint after local procaine anesthesia. Virus control birds were inoculated with virus but had no amputation. Surgical control birds were inoculated and the contralateral, noninoculated wing was amputated simultaneously with amputation of wings of experimental birds at 16 hours, 6 days, or 9 days after inoculation. Alternate amputated wings from each group were tested for virus by the wing-web technique. The others were fixed in 10 percent formalin for microscopic study. Inoculated chicks were followed daily through day 15, and 3 times weekly thereafter for at least 90 days.

On day 15, when mortality in the virus controls reached about 50 percent, and on day 25, when usually 100 percent of the controls had died, 3 to 6 birds from each experimental group, selected from the survivors by random tables, were killed. The lungs were removed from each bird for microscopic study and virus detection by the wing-web technique. Other organs were removed and studied in the same manner only if they seemed to have gross tumors.

For virus recovery the soft tissues of the wings were removed from the bones and placed in small rubber-stoppered test tubes, which were sealed with adhesive tape, labeled, and stored in the dry-ice box. Lungs and other viscera were treated similarly. For virus assay the frozen tissues were homogenized at 1:5 with 2 percent rabbit serum-saline containing 100μ/ml penicillin and 100γ/ml streptomycin. Each preparation was injected into a wing web of each of 3 chicks aged 3 to 5 days. Chicks were observed for tumor formation 3 times per week for 3 weeks. In the fluorescein-labeled antibody studies, a fluorescein-labeled chicken anti-Rous sarcoma serum globulin was used with the direct method (*2*). Effective antiserum was produced by inoculating 6- to 8-week-old chicks in the wing web with 1 cc of a 1 percent crude suspension of Rous sarcoma tissue in saline that had become noninfective during storage for at least 4 weeks at 4° C without addition of formalin or other antiviral substance. Prior to storage the minimal tumor dose of this suspension was 10^{-7} in 5-day-old chicks. The birds were reinoculated twice with the same noninfective material at 2-week intervals and were challenged after another 2-week interval by wing-web inoculation of 0.2 ml of a 1 percent fully infective virus preparation (minimum tumor dose titer 10^{-7}). Tumors did not occur. Birds were bled 2 to 4 weeks later for serum.

RESULTS

A total of 360 birds were used in 5 experiments. Since results were similar in all experiments, data are combined for analysis. Gross wing tumors were first detectable on day 7 and grew rapidly thereafter. In nonamputated controls, deaths totaled 33 percent by day 15 and reached 100 percent by day 20.

Wing Webs

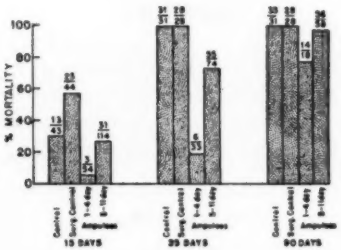
In wings amputated between 8 hours and 2 days after inoculation of virus, no microscopic pathology was observed, and no virus was demonstrated by either the wing-web technique or by the fluorescein-labeled antibody test. By day 3 there were scattered round cells that could not be identified with certainty. Virus was not detected by wing-web or fluorescein-labeled antibody tests on this day. In wings removed on the 4th day, 1 of 6 had definite sarcoma on microscopic examination. Transmissible virus was present in 1 of 9, and viral antigen was demonstrated in 4 of 6 wings by the fluorescein-labeled antibody technique (fig. 1). A detailed report of these studies is published elsewhere by Mellors and Munroe (*3*). On day 5 the incidence of microscopic tumors and infective virus in wings rose to 3 of 7 and 3 of 6, respectively. All wings removed on days 6, 7, 9, and 11 had histologically typical sarcomas, and virus was recovered from 9 of 13, 5 of 7, 6 of 6, and 5 of 5, respectively. The microscopic lesions seen before the appearance of palpable wing-web tumors consisted of one or more small, discrete foci of spindle-shaped cells in the subcutaneous space and also in the intramuscular connective tissue. When the lesion became palpable, focal lesions sometimes persisted as satellites adjacent to the predominant lesion.

Mortality

Postmortem examination of chicks that died spontaneously indicated that massive pulmonary involvement by sarcoma was the usual cause of death. Also, in the birds that were killed, lungs were the major site of gross tumor involvement.

Fifteen days after Rous sarcoma virus inoculation, the mortality of controls (text-fig. 1) was not different from birds amputated at 5 days or later, but greatly exceeded that in early amputees. Mortality of the surgical controls, amputated on day 6 or 9, was considerably greater (25/44) than the unamputated virus controls (13/43) and the experimental birds amputated at identical times (17/59).

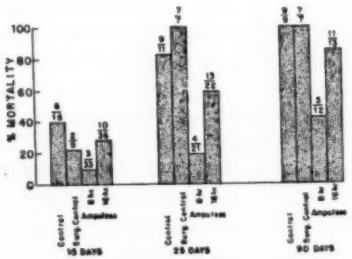
Mortality data after day 15 are biased by the removal of surviving birds to be killed on this day. They have been computed by excluding from subsequent mortality calculations all birds that were sacrificed. Thus, in text-figure 1, 31 of the 114 5- to 11-day amputee groups had died by day 15, and on that day 40 were killed for virus and histologic study. Therefore in the day-25 data the total available for analysis was reduced to 74, but deaths within this group of 74 now totaled 55. On this day



TEXT-FIGURE 1.—Combined data from all studies except 8- and 16-hour amputation. Mortality of chicks inoculated with Rous sarcoma virus, after amputation of the inoculated wing ("amputees") or uninoculated wing ("surgical controls") or intact ("controls"). The abscissa is in percentage to permit direct comparison of all groups. The actual number of chickens per group is indicated above each column. Progressive decrease in numbers per group is due to removal of birds for histologic and virus study—*see* text. Amputation prior to day 5 cured some birds. Amputation after day 5 did not cure but did increase survival through day 25. Birds that had the uninoculated wings amputated on day 6 or 9 (surgical controls) died sooner than unamputated tumor-bearing controls.

another 16 birds were killed, leaving only 58, of which only 2 survived the entire 90-day experimental period. Since mortality was unequal in these treated groups, this procedure tends to exaggerate survival in groups with low mortality and to exaggerate mortality in groups with few ultimate survivors.

Mortality of virus controls and surgical controls was 100 percent 25 days after inoculation, while birds amputated on day 5 or later had only 74 percent mortality and early amputees had only 18 percent mortality. By day 90 there were still a few survivors among the early amputees, but in late amputees mortality was nearly 100 percent.

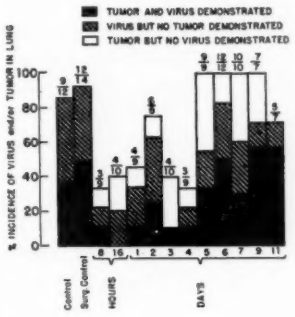


TEXT-FIGURE 2.—Mortality of chicks inoculated with Rous sarcoma virus, after amputation of the inoculated wing (amputees), or uninoculated wing (surgical controls), or intact controls. The abscissa is in percentage to permit direct comparison of all groups. The actual number of chickens per group is indicated above each column. Progressive decrease in numbers per group is due to removal of birds for histologic and virus study. Amputation even as early as 8 hours after inoculation did not prevent visceral tumors. Surgical-control (done at 8 hours) death rates were similar to those in text-figure 1.

Text-figure 2 presents similar data for the experiments in which amputations were done earlier than 1 day after virus inoculation. Mortality of the virus controls and surgical controls was similar to that in the first experiments, except that the surgical controls (16-hour) did not show high mortality at day 15. Amputation of the inoculated wing at 8 or 16 hours resulted in a lower incidence of tumor development than in the controls, but not significantly different from amputation at 1 to 4 days.

Lungs

Virus controls killed on day 15 had gross and microscopic sarcomas and infectious virus in lungs of 4 of 12 birds, and 5 had virus but no tumor (text-fig. 3). On day 15, 7 of 14 surgical controls (whose wings were amputated on day 6 or 9) had both virus and tumor in lungs, and 5 of the 14 contained virus without tumor. No survivors remained in virus control or surgical control groups by day 25. Data were obtained on both day 15 and day 25 in the experimental groups because of the better survival, but since there were no significant differences between these 2 days in incidence of either tumors or virus in lungs, data from both days are combined in text-figure 3. When amputation was on day 5 or later, over 40 percent of the birds had both virus and tumor, and nearly 100 percent had virus, tumor, or both. When amputation was on days 1 through 4, there was a lower incidence of both virus and tumor (53 percent had neither virus nor tumor), and when amputation was at 8 or 16 hours, 62 percent had neither tumor nor virus in lungs. These data are in agreement with the mortality data in indicating that progressive neoplastic disease did sometimes occur even when the inoculated wing was removed within 8 hours after injection of the oncogenic virus.



TEXT-FIGURE 3.—Incidence of demonstrable Rous sarcoma virus, tumor, or both in lungs of chicks killed 15 or 25 days after wing-web inoculation of Rous sarcoma virus. Controls were virus-inoculated but had no surgery. Surgical controls had uninoculated wing amputated at 1, 6, or 9 days after virus. Other columns refer to bird from which the wing that was inoculated with Rous virus was amputated at the indicated number of hours or days after virus injection.

It may be significant that in the experimental chickens, pulmonary sarcomas without demonstrable virus were frequent (26% in the 5- to 11-day amputees, and 16% in those prior to day 5), whereas this phenomenon did not occur among the 26 control birds. This is consistent with the assumption that these pulmonary tumors were initiated by small amounts of virus disseminated from the wing prior to amputation, since several authors have reported that tumors produced by inoculation of a small dose of virus frequently contain no detectable virus (*4–6*).

DISCUSSION

Failure to demonstrate virus at the site of inoculation until the 4th day after injection confirms previous studies in this (*7*) and other laboratories (*8–10*). Likewise no tumor cells were identifiable until the 4th day after inoculation. Yet in the present experiment, pulmonary tumors and/or virus eventually developed in 5 of the 21 birds from which the inoculated wing was removed at 8 hours after injection, and in 26 of the 65 birds with wings amputated prior to day 4. This indicates that the visceral lesions in these experimental birds cannot be attributed to metastasis of tumor cells. Presumably then, they resulted by dissemination of the causative virus.

The late appearance of demonstrable virus at the injection site, together with the demonstration of a 12- to 14-hour reproductive cycle for Rous sarcoma virus in tissue culture (*11*), suggests that 8 hours is not sufficient time for the inoculated virus to propagate and therefore that the virus disseminated to the lungs in the 8-hour amputees must be the material actually inoculated rather than newly propagated virus particles.

Surgery appeared to exhibit multiple effects in this study. It was curative in a considerable number of chicks when it removed the site of virus inoculation before the development of gross tumor, though it has been emphasized that even very early amputation was not 100 percent curative. Amputation of a wing bearing a palpable tumor, though never curative, was therapeutic in the sense of prolonging survival time. This indicates that the continuing presence of the "primary" tumor mass was prejudicial to survival—possibly because of excessive metabolic demand, or because it was a source of continuing dissemination of tumor cells, virus, or toxic products. But conversely, surgery by itself (*i.e.*, surgery that did not accomplish removal of tumor) was detrimental, as indicated by the faster mortality in surgical control groups in which the unaffected wing was amputated, as compared with unamputated controls.

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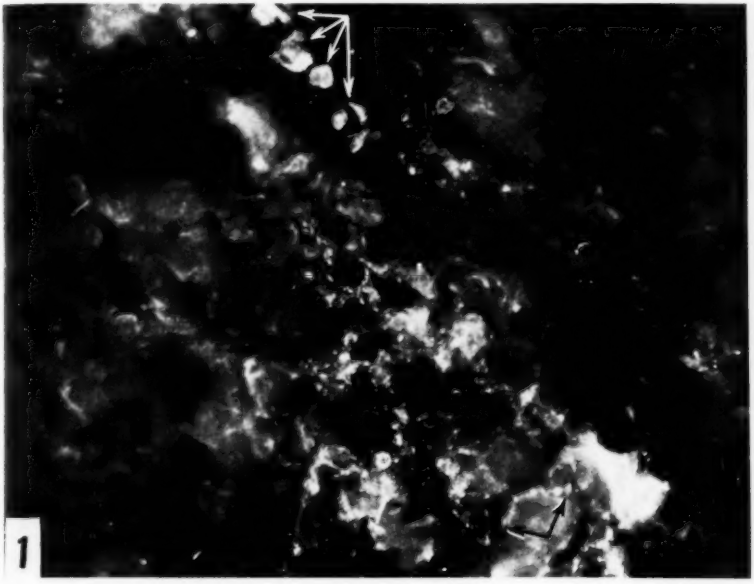
PLATE

PLATE 101

FIGURE 1.—Frozen section of wing web at 4 days after Rous virus inoculation stained by fluorescein-labeled antibody technique. At *top* are sarcoma cells containing viral antigen (*white*). At *bottom* are skeletal muscle fibers showing particulate distribution of viral antigen on sarcolemma.



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Announcements

Symposium on Host-Tumor Interactions

An international symposium entitled "Host-Tumor Interactions" has been scheduled for May 18 to 20, 1961, at the Henry Ford Hospital, Detroit, Michigan. The symposium will deal with the mechanisms of cell and tissue interactions at a basic biological level and on this level will attempt to provide an interpretation of certain aspects of neoplastic disease. Attendance at the symposium is limited to 400 invited speakers and guests. Further information may be obtained from Michael J. Brennan, M.D., Oncology Division, Secretary, Symposium on Host-Tumor Interactions, Henry Ford Hospital, Detroit 2, Michigan.

Manuscripts Approved for JNCI, January 15 to February 15, 1961

"Observations on the influence of force breeding in mice with and without the mammary-tumor agent," by John J. Bittner.

"Editorial history of the Journal of the National Cancer Institute," by Doris M. Chaney and Marie K. Murphy.

"Mammalian chromosomes *in vitro*. XIV. Genotypic replacement in cell populations," by T. C. Hsu and Donald J. Merchant.

"Myeloma globulins of plasma-cell neoplasms in inbred mice. I. Immunoelectrophoresis of serum, using rabbit antibodies prepared against microsome fractions of the neoplasms," by Michael Potter and Edward L. Kuff.

"Formation of 'hybrid'-type cells in combined cultures of two different mammalian cell strains," by G. Barski, S. Sorieul, and Fr. Cornefert.

"Some ultrastructural characteristics of a series of primary and transplanted plasma-cell tumors of the mouse," by Albert J. Dalton, Michael Potter, and Ruth M. Merwin.